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Glyphosate Susceptibility of Different Life Stages of Three Fern Species

KARLA AGUILAR-DORANTES
Instituto de Ecología, A.C., Red de Ecología Funcional, Carretera antigua a Coatepec no. 351, El Haya, Xalapa, Veracruz, C.P. 91070, México, karlachic@gmail.com

KLAUS MEHLTRETER*
Instituto de Ecología, A.C., Red de Ecología Funcional, Carretera antigua a Coatepec no. 351, El Haya, Xalapa, Veracruz, C.P. 91070, México, kmehltreter@gmail.com

MARTÍN MATA-ROSAS
Instituto de Ecología, A.C., Red de Manejo Biotecnológico de Recursos, Carretera antigua a Coatepec no. 351, El Haya, Xalapa, Veracruz, C.P. 91070, México, martin.mata@inecol.mx

HEIKE VIBRANS
Colegio de Postgraduados, Campus Montecillo, Postgrado en Botánica, km 36.5 carretera México-Texcoco, Montecillo, Texcoco, México, C.P. 56230, México, heike@colpos.mx

VALENTÍN ESQUEDA-ESQUIVEL
INIFAP, Campo Experimental Cotaxtla, km 34.5 carretera Federal Veracruz-Córdoba, Medellín de Bravo, Veracruz, C.P. 94279, México, esqueda.valentin@inifap.gob.mx

ABSTRACT.—Glyphosate, a systemic herbicide, is used against weeds in agricultural fields as well as against invasive plants in pastures, forest plantations, and urban environments. Its frequent and widespread use can negatively impact the surrounding natural non-target vegetation following the accidental drift of spray droplets, leaching, or persistence as residues in the soil. Because ferns possess a life cycle with independent sporophytic and gametophytic generations, herbicides may cause a different impact on each life stage. The objective of the present study was to evaluate the effect of four concentrations of glyphosate (0.33, 0.65, 2.72, and 10.89 g active ingredient L^{-1}) and water as control treatments on spores, gametophytes, and two sporophyte size classes of one native (*Blechnum appendiculatum* Willd.) and two introduced fern species in Mexico (*Macrothelypteris torresiana* (Gaudich.) Ching and *Thelypteris dentata* (Forssk.) E.P. St.John). Spore germination was evaluated 10 days after herbicide treatment and the percentage of remaining green tissue was measured at 7, 30, and 90 days after herbicide treatment. Plant survival was determined at the end of the experiment. Glyphosate suppressed spore germination nearly completely and increased tissue discoloration of all green life stages at higher concentrations and after longer time intervals. After 7 and 30 days, small sporophytes of all three species were significantly more discolored than gametophytes and large sporophytes at concentrations $\leq$ 0.65 g a.i. L^{-1}, although after 90 days differences among life stages were no longer significant, and no life stage survived at concentrations $\geq$ 2.72 g a.i. L^{-1}. At the lowest concentration (0.33 g a.i. L^{-1}), however, 50–69% of the plants of all three species and life stages survived after 90 days post-treatment. *Macrothelypteris torresiana* was significantly more tolerant to glyphosate than the other two species during the first 30 days after treatment, mainly because of its less damaged small sporophytes, perhaps due to a thicker waxy cuticle that may initially reduce herbicide absorption in this species. We conclude that even glyphosate concentrations of 0.33 g a.i. L^{-1} may negatively impact natural spore banks of ferns and result in mortality of 31–50% of all green life stages. Such negative effects may also eliminate at least the most susceptible fern species in habitats that are frequently exposed to such glyphosate concentrations.

KEY WORDS.—gametophyte, herbicides, non-target plants, spore bank, sporophyte

* Corresponding author.

Glyphosate (N-(phosphonomethyl)-glycine) is one of the most widely used non-selective herbicides on agricultural lands and forest plantations as well as swamps, parks, and gardens (U. S. Environmental Protection Agency, 1993; Cox, 2000; Kogan and Alister, 2010, Henderson *et al.*, 2010). As a systemic herbicide, glyphosate is absorbed mainly through leaves and axes and translocated via the vascular bundles to shoots and roots (Harker and Dekker, 1988). Glyphosate is also absorbed by the soil, depending on its structure and composition (Gimsing and Borggaard, 2002), where it can damage the microbiota, including nitrogen-fixing bacteria (Santos and Flores, 1995). Glyphosate degradation in the environment is mainly the consequence of the activity of the bacteria *Pseudomonas fluorescens* and *Acetobacter* sp. (Moneke *et al.*, 2010). In soils of natural environments, glyphosate has a mean half-life of 47 days (range between 2 and 197 days; Giesy *et al.*, 2000). Consequently, glyphosate residues can be detected for months in treated areas. Aparicio *et al.* (2013) found glyphosate concentrations in agricultural soils of up to 0.19 mg kg^{-1} soil at 188 days after herbicide application, and Peruzzo *et al.* (2008) reported concentrations of 1–5 mg kg^{-1} in soils at 150–1500 m distance from soybean fields at 30 days after herbicide application.

The prolonged, frequent, and widespread use of herbicides causes multiple effects on weeds and non-target plants. Although glyphosate is quickly bound to clay minerals of the soil matrix (Zhou *et al.*, 2004), non-target species that grow in the close vicinity of areas of herbicide application may be negatively affected after accidental exposure by spray drift (de Snoo and van der Poll, 1999; Rodriguez and Jacobo, 2010), by leaching after heavy rainfall (Edwards *et al.*, 1980), and by translocation from shoots of target plants to the rhizosphere (Neumann *et al.*, 2006). Low glyphosate concentrations can cause nutrient deficiencies in non-target plant species (Dick and Quinn, 2010) because they restrict absorption of some mineral nutrients (Neumann *et al.*, 2006).

Between 2005 and 2009, Mexico reported an annual use of 44,000–57,000 tons of herbicidal active ingredients (FAO, 2014). This level of herbicide use may negatively affect Mexico's plant diversity, which consists of 21,841 native angiosperms (Villaseñor and Ortiz, 2014) and 987 ferns (Mickel and Smith, 2004). Ferns have been recognized as good indicators of altered light conditions and decreased relative humidity following environmental disturbance and changes in land use (Mehltreter, 2008). Fern growers know about the susceptibility of many ornamental fern species to pesticides and fungicides and consequently recommend their use on ferns only at reduced concentrations (Jones, 1987, Rickard, 2000). In contrast, land managers search for effective herbicides to control some of the problematic weedy ferns such as *Pteridium aquilinum* (L.) Kuhn and *Lygodium microphyllum* (Cav.) R.Br. (e.g., Hutchinson and Langeland, 2011). Asulam, a herbicide used to control *Pteridium aquilinum*, however, damages non-target ferns considerably more than *P. aquilinum* (Keary *et al.*, 2000). Rowntree and Sheffield (2005) found that four of eight fern species were severely damaged and two of them killed one year after asulam treatments at concentrations ten times lower than the recommended field dose. Consequently, we suggest that some fern species

might be also very susceptible to glyphosate and may be useful bioindicators for herbicide contamination and the presence of glyphosate residues.

Most of the former results were obtained on a single life stage, but ferns possess a life cycle with unicellular spores as dispersal units, and two independent, multicellular photosynthetic generations, the small haploid gametophyte and the larger diploid sporophyte. The survival of fern species will depend on the herbicide susceptibility of each of the three life stages and fern populations could vanish if the life cycle should be disrupted by the extinction of the most susceptible life stage. Thus, the objective of this study was to investigate the effect of different concentrations of glyphosate on spores, gametophytes, and small and large sporophytes of three fern species. We assumed that the selected fern species might suffer accidental exposure to herbicides in their natural habitats, because they are abundant along trails, roadsides, forest margins and borders of agricultural lands, where herbicides are applied. Two of the three selected species (*Macrothelypteris torresiana* and *Thelypteris dentata*) belong to the 22 species introduced into Mexico (Tejero-Diez and Torres-Diaz, 2012), and both are weedy and more abundant in ruderal habitats than the native species (*Blechnum appendiculatum*). Thus, we expected (1) that the two introduced species might be less susceptible to glyphosate than the native fern species. We also expected (2) that gametophytes would be more susceptible to glyphosate than the other life stages because of their thin laminar structure and lack of a cuticle.

Materials and Methods

Study species.—Three fern species were selected for this study because they grow commonly in disturbed habitats and ruderal sites in close vicinity to areas where herbicides are applied, but where they are not the target of the herbicide treatments. *Blechnum appendiculatum* Willd. (Blechnaceae) is a native fern ranging from the southern United States to Argentina. It has short, erect rhizomes, and chartaceous, glabrous, pinnate leaves of 10–50 cm length and 4–10 cm width. *Macrothelypteris torresiana* (Gaudich.) Ching and *Thelypteris dentata* (Forssk.) E.P. St. John (Thelypteridaceae) are fast growing species that were introduced into Mexico about 50 years ago and became naturalized (Mickel and Smith, 2004). *Macrothelypteris torresiana* has short-creeping rhizomes and herbaceous, hairy, glandular, 2-pinnate-pinnatifid leaves of 60–150 cm length and 50 cm width. Young stipes and blades are glaucous because they are covered by a thin coat of wax. *Thelypteris dentata* has short-creeping rhizomes and herbaceous, hairy, 1-pinnate-pinnatifid leaves of 40–140 cm length and 15–35 cm width.

Spore collection.—Mature fertile leaves of each of the three study species were collected to obtain spores. Collections were repeated three times per year from several individuals growing along trails of cloud forest fragments in Huatusco and Xalapa, Veracruz, Mexico. Leaves of all individuals of the same species and collection date were combined into a single composite sample. The fertile leaves were washed with soapy water and dried in open paper bags

at room temperature. Spores were stored for 2 to 12 weeks before starting any of the experiments. Voucher specimens of each species were deposited in the herbarium of the Instituto de Ecología, A. C. (XAL).

Spore sieving and sterilization.—Spores from each location were sieved through a mesh with a pore size of 50 μm. After sieving, portions of approximately 1 mg of spores (ca. 20,000 individual spores) were packed in filter paper envelopes (Whatman No. 5 or 5.5 diameters) and those were immersed in 70% (v/v) ethanol for 1 min, in distilled water and hypochlorite solution (0.5% w/v) for 5 min, and in distilled water with two drops of Tween-80 per 200 mL (Sigma, St. Louis, MO) for 5 min. Subsequently envelopes were rinsed three times for 3–5 min with sterile distilled water under aseptic conditions to reduce contamination of agar plates with fungi, bacteria, and algae (Fernández and Revilla, 2003).

Cultivation medium.—Murashige and Skoog (MS) medium was prepared in distilled water (Murashige and Skoog, 1962) and titrated to pH 5.7 using concentrated hydrochloric acid. After dissolving 7.0 g of agar L^{-1} the solution was autoclaved for 20 min at 120°C and 1.2 kg cm^{-2} pressure. After 20 min of cooling, agar–nutrient solution was added to Petri dishes and test tubes under a laminar flow hood. The medium was stored at 4°C until inoculation.

Cultivation of spores, gametophytes, and small sporophytes.—Under a laminar flow hood, sterilized spores were transferred from one filter envelope to a petri dish and incubated for 60 days in a growth chamber under cool-white fluorescent light (50 μmol m^{-2} s^{-1}) with a photoperiod of 16 h light/8 h darkness and a temperature of 25°C ± 1°C. After subcultivation of approximately 200 gametophytes for another 60 days in petri dishes, two large gametophytes were transferred into each of 16 test tubes (2.5 cm diameter and 15 cm height) with solid MS media, whereas all gametophytes with attached sporophytes were subcultivated for another 180 days in pots of 11.5 cm diameter and 7.5 cm height. Finally, two young sporophytes with leaves of 8–10 cm length were transplanted into each of 16 test tubes (2.5 cm diameter and 15 cm height) with solid MS media.

Cultivation of large sporophytes.—Larger sporophytes of *Thelypteris dentata* (25–30 cm height) were obtained from spore culture, whereas plants of *Blechnum appendiculatum* (20 to 35 cm) and *Macrothelypteris torresiana* (30 to 40 cm) were obtained from field collections. Sporophytes with at least three leaves were transplanted individually in 1 L pots of 13 cm diameter containing 2/3 humus and 1/3 sand and acclimatized for 1 month in the greenhouse with 55% shade and an average temperature of 19.8°C. The latter data are averages of daylight and temperature measured for a week with a LI-190SA Quantum Sensor and 1000–1016 temperature sensor (Li-Cor), connected to a datalogger (LI-1000, Li-Cor, Inc. Lincoln, Nebraska, USA).

Glyphosate treatments.—One liter of commercial stock solution of glyphosate, containing 363 g of the active ingredient (a.i.) glyphosate acid, was sterilized with sterile filtration units (Nalgene, Bottletop filters, 50 mm diameters) connected to a vacuum pump. This stock solution was diluted with water to concentrations of 0.09, 0.18, 0.75 and 3.00% corresponding to 0.33,

0.65, 2.72, and 10.89 g a.i. L^{-1}. The latter two concentrations corresponded to the lower and upper limit of commonly applied glyphosate concentrations in the field, depending on the crop and weed species (e.g., Duke, 2011). The former two concentrations correspond to the predicted glyphosate levels remaining after 2 and 3 half-lives of an original application of 2.72 g a.i. L^{-1} have elapsed, respectively. For each species and glyphosate concentration, herbicide solutions were applied 1 day after spore inoculation to 16 petri dishes (0.4 ml) and 7 days after transfer of gametophytes (0.4 ml) and young sporophytes into 16 test tubes (0.8 ml). Control treatments for each species and life stage were performed simultaneously with herbicide treatments. Sterile water rather than herbicide solutions was applied to control treatments of spores in Petri dishes and gametophytes and young sporophytes in test tubes with 16 replicates each. After 24 h of incubation in the growth chamber, spores, gametophytes and young sporophytes of all treatments were transferred into new MS medium, free of glyphosate and incubated in the growth chamber. For each species, 16 large sporophytes were treated with 25 ml of glyphosate solutions by means of a spray bottle taking care that all leaves were completely wetted. Glyphosate is absorbed by the leaves and axes and inhibits the shikimate pathway by blocking the biosynthesis of aromatic amino acids (Zablotowicz and Reddy, 2004), alkaloids, flavonoids, lignins and cyanogenic glycosides (Franz *et al.*, 1997). As a consequence, the herbicide arrests cell division and growth, causes chlorosis, damages the cell membrane (Hoagland and Duke, 1982; Caseley *et al.*, 1993; Bott *et al.*, 2008) and results in plant death.

Evaluation of treatments.—The treatment effect on spores was determined as the relative percentage of germination. At 7 and 30 days after the herbicide treatment, germinated and non-germinated spores were counted in three grid cells of 1 cm^2 under a microscope at 100X magnification. Germination rates were calculated by dividing the number of germinated spores by the total number of spores in the three grids. Spores were counted as germinated if the primary prothallial cell had emerged from the spore. For all gametophytes and sporophytes, the relative percentage of remaining green tissue was estimated for each individual plant at 7, 30 and 90 days after treatment and assigned to one of seven classes (0% = no green tissue, 1–5%, 6–25%, 26–50%, 51–75%, 76–99% and 100%, i.e., no decolorized tissue). For all calculations the class median was used. Estimates for gametophytes and small sporophytes were averaged for each test tube before further statistical analyses.

Statistical analyses.—Glyphosate susceptibility was analyzed with a three-way ANOVA (3 species, 3 life stages and 5 concentrations) of repeated measures (7, 30 and 90 days post-treatment), followed by a Tukey multiple comparison procedure ($p < 0.05$). Means of percentage values of green tissue were normalized by arcsine-square-root transformation before the analyses. Differences in survival among species and life stages were analyzed by Chi-square test. Statistical tests were performed with Statistica version 7.1 (StatSoft, Tulsa, Oklahoma, USA).

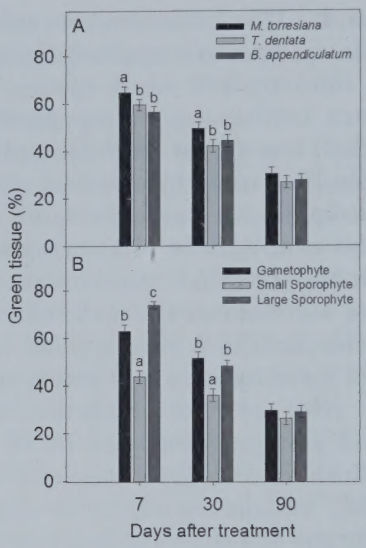

FIG. 1. Relative amount of remaining green tissue (%) of A) species and B) life stages after glyphosate treatment. Means ± S. E.

RESULTS

Glyphosate susceptibility of spores.—Glyphosate inhibited spore germination completely with the single exception of *Macrothelypteris torresiana* of which 0.31% ± 0.3% of spores germinated at the lowest glyphosate concentration of 0.33 g a.i. L^{-1}, although control treatments of all three species exhibited high germination rates (*B. appendiculatum*: 96.1 ± 1.7%, *T. dentata*: 96.9 ± 1.2%, and *M. torresiana*: 98.6 ± 0.7%).

Glyphosate susceptibility of different green life stages and species.—Glyphosate susceptibility varied significantly among species (ANOVA, $F_{2,675} = 20.59$, $p < 0.001$; Fig. 1A), and among green life stages ($F_{2,675} = 100.14$, $p < 0.001$; Fig. 1B) at distinct glyphosate concentrations ($F_{4,675} = 1977.58$, $p < 0.001$) and over time ($F_{4,675} = 1048.66$, $p < 0.001$; Table 1). Our first hypothesis was partially confirmed, because one of the introduced species, *Macrothelypteris torresiana* was significantly less susceptible than the other two species, although glyphosate susceptibility of the second introduced species, *Thelypteris dentata*, did not differ from that of the native fern species *Blechnum appendiculatum* (Tukey, $p < 0.05$, Fig. 1A). These differences were due to the lower susceptibility of *M. torresiana,* especially its more tolerant young sporophyte, during the first 30 days after treatment at glyphosate concentrations of 0.33–0.65 g a.i. L^{-1} (Fig. 2). However, discoloration of all life stages of all species increased with higher glyphosate concentrations and longer time intervals after treatment (Fig. 2). In contrast to our second hypothesis, gametophytes were not the most susceptible life stage but exhibited intermediate levels of glyphosate susceptibility. Damage to gametophytes appeared more slowly than in small sporophytes but discoloring was

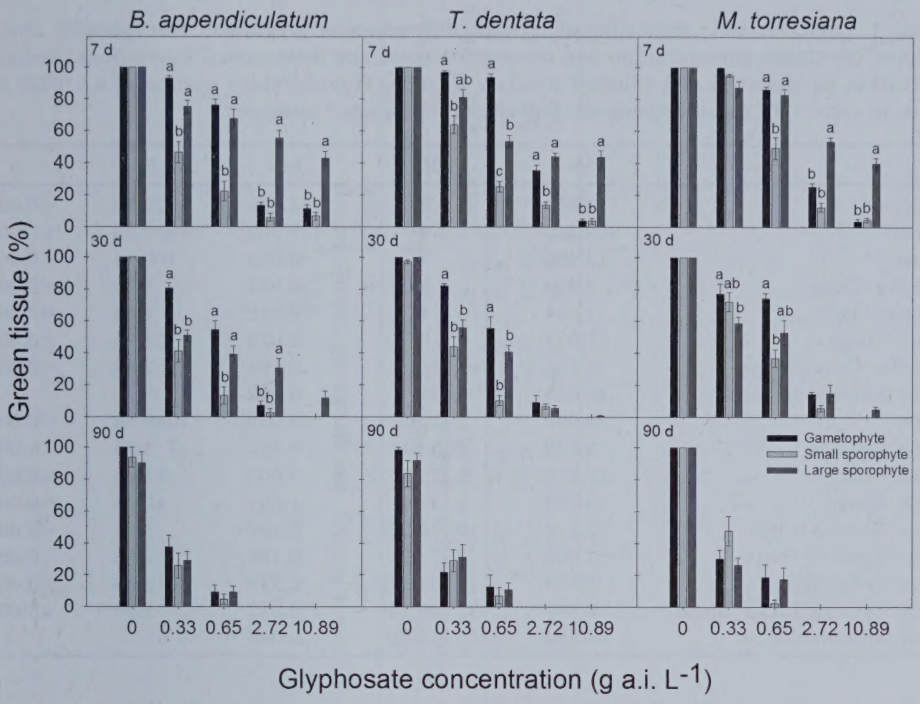

FIG. 2. Relative amount of remaining green tissue (%) of life stages of three fern species at 7, 30 and 90 days after glyphosate treatment. Means ± S. E.

faster than observed in large sporophytes (Fig. 1B). At higher concentrations ($\geq$ 2.72 g a.i. L^{-1}) tissues of large sporophytes remained 40–60% green for seven days after treatment whereas the other life stages were significantly more discolored (Tukey, $p < 0.05$), and all life stages were increasingly damaged after 30 days and completely dead 90 days after treatment (Fig. 2). At lower concentrations ($\leq$ 0.65 g a.i. L^{-1}), large sporophytes as well as gametophytes were significantly less discolored than young sporophytes until 30 days after treatment (Tukey, $p < 0.05$), although 90 days after treatment all three life stages were similarly damaged, but with survival rates ranging between 12.5 and 37.5% at concentrations of 0.65 g a.i. L^{-1} and between 50.0 and 68.8% at 0.33 g a.i. L^{-1} (Table 2). Survival rates after 90 days did not differ among species nor among life stages (all Chi-square values $\leq$ 1.12, $p > 0.05$, df = 4).

DISCUSSION

In this study, we found no consistent support for our first hypothesis that introduced fern species are less susceptible to glyphosate than native fern species, although the least susceptible species in our sample — *Macrothelypteris torresiana* — is indeed introduced. Also, our results did not support the second hypothesis, that gametophytes would be the most susceptible life stage to glyphosate. Instead, the young sporophyte was the most susceptible

TABLE 1. ANOVA table indicating significant differences of glyphosate susceptibility among species, life stages, concentrations and observation times and their mutual interactions. Reduced df (within parentheses) and adjusted p-values ([a]) using Huynh-Feldt's epsilon of 0.910929 are given to correct for non-independence (sphericity) of repeated measures.

	SS	df	MS	F	p
Species	2.859	2	1.429	20.59	<0.001
Conc.	549.210	4	137.302	1977.58	<0.001
Stage	13.906	2	6.953	100.14	<0.001
Species*Conc.	2.939	8	0.367	5.29	<0.001
Specie*Stage	1.354	4	0.339	4.88	<0.001
Conc.*Stage	15.027	8	1.878	27.05	<0.001
Species*Conc.*Stage	3.108	16	0.194	2.80	<0.001
Error between subjects	46.865	675	0.069		
Time	88.096	2 (1.82)	44.048	1048.66	<0.001[a]
Time*Species	0.629	4 (3.64)	0.157	3.74	0.007[a]
Time*Conc.	27.238	8 (7.29)	3.405	81.06	<0.001[a]
Time*Stage	6.763	4 (3.64)	1.691	40.25	<0.001[a]
Time*Species*Conc.	2.192	16 (14.57)	0.137	3.26	<0.001[a]
Time*Species*Stage	1.001	8 (7.28)	0.125	2.98	0.004[a]
Time*Conc.*Stage	8.708	16 (14.57)	0.544	12.96	<0.001[a]
Time*Species*Conc.*Stage	2.669	32 (29.15)	0.083	1.99	<0.001[a]
Error within subjects	56.705	1350 (1230)	0.042		

life stage. As expected, at higher glyphosate concentrations, tissue discoloration continued until the death of all species across all life stages. At lower glyphosate concentrations, a small percentage of individuals of all three species survived, but did not show signs of recovery after 90 days.

Glyphosate effect on spore germination.—Glyphosate nearly completely inhibited spore germination of the three studied species even at the lowest applied concentration (0.33 g a.i. L^{-1}). This result was unexpected because glyphosate is supposedly a post-emergence herbicide (Kogan and Alister, 2010) that should not have considerable effect on pre-emergent spores. Considerably higher concentrations of glyphosate (56.4 and 243.6 g a.i. L^{-1}) were necessary to inhibit 50% and 95%, respectively, of spore germination of *Lygodium microphyllum* (Cav.) R. Br. (Hutchinson and Langeland, 2011). However, the increased herbicide tolerance of *L. microphyllum* might be a consequence of the spore size, which is 2–3 times larger than the three species studied here (Gómez-Noguez, 2012; Giacosa *et al.*, 2013). However, spore size alone may not necessarily reduce herbicide tolerance; megaspores of *Regnellidium diphyllum* Lindm. are ca. 4–6 times larger than spores of *Lygodium*, but were susceptible to glyphosate concentrations as low as 0.0048 g a.i. L^{-1}, and showed reduced germination of ca. 20% at glyphosate concentrations of 0.0192 g a.i. L^{-1} (Droste *et al.*, 2010). Currently, too few fern species have been evaluated for their susceptibility to herbicide to draw conclusions about which spore characteristics might be correlated with herbicide susceptibility. If other fern species are as susceptible to low glyphosate concentrations in the soil as our three study species in the

TABLE 2. Survival (%) of green life stages of three fern species at 90 days after glyphosate treatment.

	B. appendiculatum					*T. dentata*					*M. torresiana*				
	Glyphosate concentration (g a.i. L^{-1})														
Life stages	0	0.33	0.65	2.72	10.89	0	0.33	0.65	2.72	10.89	0	0.33	0.65	2.72	10.89
Gametophytes	100.0	68.8	25.0	0.0	0.0	100.0	50.0	18.8	0.0	0.0	100.0	68.8	31.3	0.0	0.0
Small Sporophytes	93.8	56.3	12.5	0.0	0.0	87.5	62.5	12.5	0.0	0.0	100.0	68.8	6.3	0.0	0.0
Large Sporophytes	93.8	68.8	37.5	0.0	0.0	100.0	62.5	37.5	0.0	0.0	100.0	68.8	31.3	0.0	0.0

laboratory, natural soil spore banks may suffer significant decreases in viability when exposed to glyphosate. Such a negative impact may especially affect rare and threatened species, for which the natural spore bank has been considered a possible source for the recovery of viable spores for species conservation programs (Dyer, 1994). Field studies, similar to those on the effect of glyphosate on seed banks (Rodriguez and Jacobo, 2013), are necessary to elucidate the correlation between the extremely variable half-life of glyphosate (2–197 days; Giesy *et al.*, 2000) and viability of spore banks in a variety of threatened as well as invasive fern species in different soils and climates.

Glyphosate effect on green life stages of ferns.—Plant age has been shown to affect herbicide susceptibility (Marrs *et al.*, 1991). Consequently, we also expected that structural differences among gametophytes, small sporophytes, and large sporophytes might be correlated with different glyphosate susceptibility. However, the few available studies of chemical control of multiple life stages of invasive ferns do not support this hypothesis. For example, ninety-five percent of gametophytes of *L. microphyllum* died at similar concentrations of metsulfuron-methyl in the laboratory (26.4 mg L^{-1}; Hutchinson and Langeland, 2013) as sporophytes in the field (30 mg L^{-1}; Hutchinson and Langeland, 2012). In the present study, all life stages of our three fern species experienced similar damage from glyphosate at 90 days post-treatment. However, during the first 7–30 days, young sporophytes of all three species were damaged faster by glyphosate than gametophytes and large sporophytes, and these differences were most apparent at lower glyphosate concentrations (0.33 and 0.65 g a.i. L^{-1}). These results may indicate that life stages of ferns differ mainly in their absorption rate of herbicides. Younger plants have a less developed cuticle than older plants, which makes it easier for water soluble herbicides, such as glyphosate, to penetrate the cuticle (Harker and Dekker, 1988), resulting in a more rapid response in small sporophytes. On the other hand, plants may absorb and translocate glyphosate 3–14 days after treatment (Tworkoski and Sterrett, 1987), so that larger sporophytes with more developed cuticles continue to take up glyphosate over time. In addition, larger sporophytes intercept greater doses of herbicide with their larger leaves and translocate it throughout the entire plant (Harker and Dekker, 1988). The larger amount of intercepted, although slowly absorbed, glyphosate, may explain why large sporophytes in the present study experienced similar mortalities after 90 days than the other two green life stages.

In practice, agronomists often add coadjutants such as surfactants to herbicide solutions to increase their absorption rates. Consequently, we expect that these mixtures would likely accelerate glyphosate uptake and reduce the observed differences of discoloration among life stages. Gametophytes should absorb herbicides even faster than sporophytes because they do not have cuticles and they possess a large surface area/volume ratio favoring herbicide absorption. However, gametophytes were affected more slowly by glyphosate than young sporophytes. One possible explanation for this pattern is that gametophytes are more tolerant to glyphosate because of a slower growth rate,

which demands fewer nutrients for which damaging effects only become apparent after a longer time interval following glyphosate treatment. Thus, glyphosate application at high concentrations ($\geq$ 2.72 g a.i. L^{-1}) should not be expected to cause changes in the demographic structure of fern populations because all green life stages were similarly damaged after 90 days. However, at low glyphosate concentrations ($\leq$ 0.65 g a.i. L^{-1}) such as may occur in the vicinity of agricultural fields, the demographic structure of fern populations may suffer from an elimination of young sporophytes.

Glyphosate effect on different fern species.—Because crop species differ significantly in their glyphosate susceptibility (Boutin and Rogers, 2000), it is not surprising that glyphosate also affects non-crop plant species differentially (Strandberg *et al.*, 2012). Consequently, plant communities adjacent to agricultural fields and forest plantations managed with herbicides have been reported to change (Coutris *et al.*, 2011). For instance, grassland communities that were exposed to glyphosate had a larger proportion of annual relative to perennial species than untreated controls (Rodriguez and Jacobo, 2010). Ferns have been reported as moderately to highly sensitive species to glyphosate. In the aquatic environment, *Azolla filiculoides* Lam. was one of the most sensitive species to a mixture of three herbicides at concentrations of 0.0000006–0.0006 g a.i. L^{-1} (Coutris *et al.*, 2011). In Chilean forest plantations, *Polystichum munitum* (Kaulf.) C. Presl was less susceptible to glyphosate concentrations of 12.5–14.4 g a.i. L^{-1} (2.5-2.88 kg ha^{-1}; Kogan and Alister, 2010) than the invasive *Lygodium microphyllum* in Florida to concentrations of 5.2–10.4 g a.i. L^{-1} (Hutchinson and Langeland, 2012). Of the three fern species studied here, *Macrothelypteris torresiana* was the least susceptible to glyphosate, perhaps because of the presence of epicuticular waxes on its leaves, which give young leaves a glaucous green appearance (Mickel and Smith, 2004). Epicuticular waxes have been reported to slow herbicide absorption (Kirkwood, 1999), because they act as a barrier for herbicide uptake. Consequently, we assume that species such as *M. torresiana* may have a selective advantage against other species when exposed to sublethal glyphosate concentrations. Because of the very limited taxonomic diversity that has been studied to date, i.e. the three studied species here as well as six of the eight species of Rowntree and Sheffield (2005) all belonged to the clade of eupolypods II (Schuettpelz and Pryer, 2007; Rothfels *et al.*, 2012), we cannot assume any phylogenetic pattern of glyphosate susceptibility.

Conclusion.—Even low concentrations of glyphosate ($\leq$ 0.65 g a.i. L^{-1}) may severely damage ferns at all life stages, including ungerminated spores. These impacts may become apparent in areas adjacent to agricultural fields that are treated repeatedly with glyphosate because ferns growing in such areas are prone to accidental exposure, surface water run-off, and the accumulation of herbicide residues. For aerial spraying of herbicides, buffer zones of up to 180 m have been recommended to avoid herbicide impact on natural vegetation depending on herbicide type and concentrations (Marrs and Frost, 1996). Because fern species differ considerably in glyphosate susceptibility, changes in species composition, especially of ruderal non-target vegetation are

expected to favor a few, more tolerant species at the cost of more susceptible ones. A phylogenetic pattern of herbicide susceptibility cannot be assumed because a very limited taxonomic diversity has been studied to date. Future studies are required to evaluate herbicide effects in the field, and track their long-term effects on fern communities.

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Use of Gemma Characters to Identify North American *Huperzia* (Lycopodiaceae)

Arthur V. Gilman*
Gilman & Briggs Environmental, 1 Conti Circle, Suite 5, Barre, VT 05641 U.S.A.
avgilman@together.net
Weston L. Testo
Pringle Herbarium, Department of Plant Biology, University of Vermont, 27 Colchester Avenue, Burlington, Vermont 05405, U.S.A. wtesto@uvm.edu

Abstract.—All North American firmosses (*Huperzia*: Lycopodiaceae) produce highly specialized vegetative propagules known as gemmae. Though gemmae are of interest to morphologists, they have been used only rarely as aids in identification. To improve understanding of their variation among North American species and to explore their systematic utility we surveyed gemmae of all species in North America north of Mexico and provide measurements, comparative descriptions, and images. Several characters of the gemmae, including their size, shape, and morphology of the leaves that comprise them vary considerably across the species studied and can be used to distinguish species. A dichotomous key based on gemma characters is provided, and several notable range expansions are reported. We anticipate this study will help resolve confusion regarding the identity of North American *Huperzia* species, particularly among the taxa in the northern and western regions of the continent, which remain poorly understood.

Key Words.—Firmosses, morphology, gemmae, bulbils, lycophytes

The temperate firmoss genus *Huperzia* Bernh. is characterized in part by their terrestrial growth and production of gemmae and is sister to the tropical, non-gemmiferous firmosses, *Phlegmariurus* Holub (Wikström and Kenrick, 1997, 2000). Identification of North American species continues to pose a significant challenge (Wagner and Beitel, 1993; Haines 2003), which is based on their simple body plan and paucity of morphological structures, and intraspecific variation. This, in turn, results in an incomplete understanding of species' ranges. There has been considerable difficulty in identification of specimens, especially those from northern and western parts of the continent since the most recent revision of the North American species in the Flora of North America (Wagner and Beitel, 1993). That revision was, essentially, an unfinished work because its primary author, Beitel, passed away before he had completed field work and other studies in his doctoral program. Although the most comprehensive treatment of North American firmosses to date, it failed to resolve some uncertainties. As a result, more recent North American authors (Tzvelev, 2003; Aiken *et al.*, 2007; Dignard, 2014) have subsequently recognized taxa and distributions in North America inconsistent with those

*Corresponding author.

of Wagner and Beitel (1993), relying instead on European and Asian authorities (e.g. Rothmaler, 1993; Zhang and Iwatsuki, 2013).

Although it was stated by Wagner and Beitel (1993) that characters of the gemma, including "size, overall outlines, and gemma leaf shapes" were diagnostic, they published only limited data, and did not discuss or illustrate gemma outlines or leaf shapes. Various authors have used the size and general shape of lateral leaves, along with the shape of their apices, to discriminate species pairs on a case-by-case basis (Butters & Abbe, 1953; Waterway, 1986; Beitel and Mickel, 1992; Brunton *et al.*, 1992) or in regional treatments (Wagner *et al.*, 1999; Palmer, 2003; Haines, 2003). However, to date, no comprehensive treatment has been available. Our goals in this paper are to survey the morphology of gemmae of all North American species and to demonstrate their utility for identification. We provide images, morphometrics, and written descriptions.

Groundplan description.—Firmoss gemmae are vegetative reproductive structures distributed along or near the summit of the annual shoot growth increments. Smith (1920) provided a general description which, for clarity in our descriptions of individual taxa, we enlarge here.

Development of an individual gemma begins with the formation of a specialized shoot known as a gemmiphore. This shoot comprises six leaves, decussately arrayed in three pairs that, together, form a cupule in the center of which forms the gemma. The proximal gemmiphore leaf is enlarged and forms a rigid platform on which the mature gemma is horizontally positioned, its broadest surface presented upward. When fully grown, an abscission layer is formed between the gemmiphore and gemma. The gemma is readily detached and falls or is projected from the gemmiphore, sometimes by the force of raindrops (Victorin, 1925).

The basic structure of the gemma is relatively simple (Fig. 1). Like the gemmiphore, the gemma is a modified branch that comprises six leaves inserted on a very short axis and, distal to these, a minute terminal shoot with spirally inserted leaves (Bierhorst, 1971; Testo and Gerdes, 2015). Stevenson (1976) found that the six outer leaves are in a low parastichous spiral but, for our purposes, it is sufficient and preferable to describe them as three opposite pairs, each perpendicular to the next pair. From the outside, only five are normally visible although, rarely, the distal tip of the sixth leaf may also be visible. The terminal shoot is enclosed by the six outer leaves and is always hidden from view. When a detached gemma falls to the ground, the terminal shoot begins to grow to form the main axis of a new, independent plant.

A mature gemma is normally positioned horizontally relative to the axis of the main shoot on which it occurs. Thus the gemmae leaves are here described as if viewed from above or below and the terms "abaxial" and "adaxial" are used in relation to the axis of the gemma.

The leaves of the proximal pair, hereafter referred to as the "upper" and "lower" leaves (Fig. 1) are appressed to the other leaves so only their abaxial surfaces are visible. They are conspicuously shorter than those of the middle

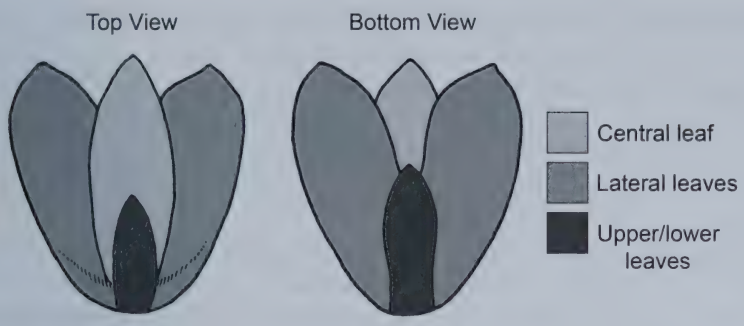

FIG. 1. Generalized diagram of the structure of a *Huperzia* gemma. Hatched lines indicate the twisted base of the lateral leaves. The lower leaf of the distal pair is not shown.

and distal pairs. They are of different size and shape relative to each other and vary from oblong to narrowly elongate-triangulate or lanceolate.

The middle pair, hereafter referred to as the "lateral" leaves (Fig. 1) are the largest and most conspicuous of the gemma leaves. They are presented in a different rank than the other gemma leaf pairs and twisted 90° into the plane of the other two pairs and are arrayed so that their adaxial surfaces are visible when viewed from above. The lateral leaves are asymmetrically elliptical to obovate with their outer margins typically more strongly curved than their inner margins. Their apices vary from obtuse to acute, and in some species they possess a mucronulate tip.

The distal pair is aligned in the same rank as the proximal leaf pair. Typically, only the upper leaf of this pair is visible. It is referred to hereafter as the "central" leaf (Fig. 1) because it is presented in the center of the gemma above and between the two lateral leaves. Its shape varies from broadly oblong to broadly pandurate, with an acute or obtuse apex. Because the lower leaf of the distal pair is usually hidden, it is not readily available for observation and will not be discussed further.

MATERIALS AND METHODS

In their Flora of North America treatment, Wagner and Beitel (1993) recognized seven species of *Huperzia* in continental North America north of Mexico. We also recognize *Huperzia arctica* (Gross. ex Tolm.) Sipl., a circumarctic species not mentioned in their treatment; and following Haines (2003) we use the older name *Huperzia appressa* (Desv.) Á. Löve & D. Löve for the taxon treated by Wagner and Beitel (1993) as *Huperzia appalachiana* Mickel & Beitel. For our study, species determinations were based on comparison to type material and to descriptions by Tolmachev (1960), Wagner and Beitel (1993), and Haines (2003).

We examined 1103 gemmae from 150 specimens (Appendix 1) from the following herbaria: ALA, GH, NY, VT and WTU. Our sampling approach incorporated specimens from across each species' range in North America and included approximately equivalent representations of both gemmae and speci-

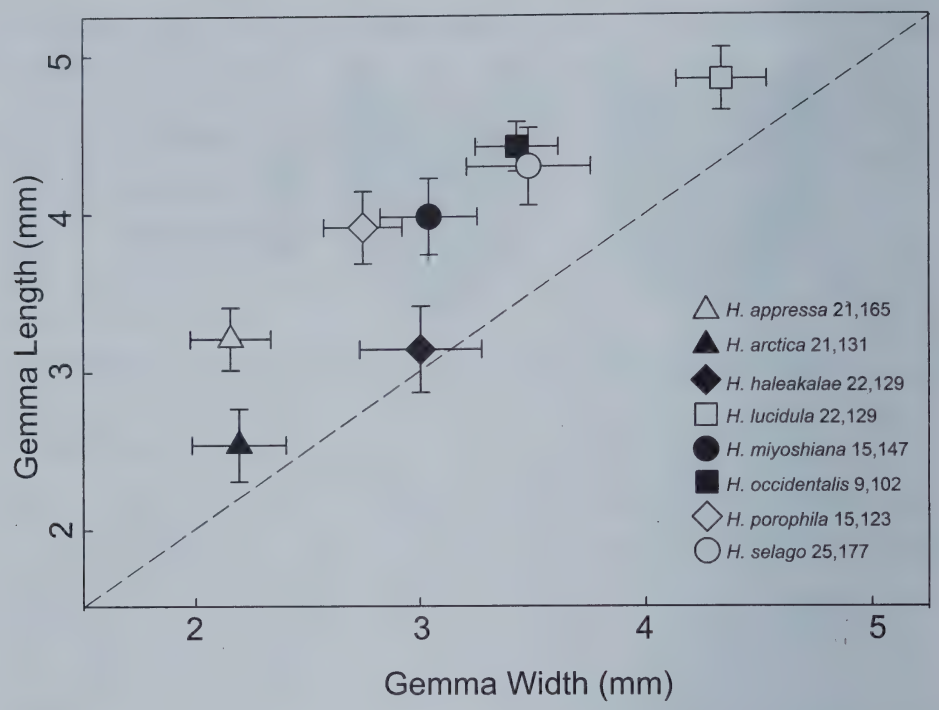

FIG. 2. Scatter plot showing overall length and width of North American *Huperzia* gemmae. Error bars represent ± 1 standard deviation. The dashed line marks a 1:1 length:width ratio.

mens across the species studied. The number of specimens examined per species ranged from 9 for *H. occidentalis* to 25 for *H. selago*; the number of gemmae examined per species ranged from 102 for *H. occidentalis* to 177 for *H. selago*. In general, 6–12 gemmae from each specimen were obtained from the specimen's damage packet or removed from the plant using a dissecting probe; for some specimens, fewer gemmae were available for measurement. Care was taken to use fully formed gemmae, but to exclude over-mature gemmae, which sometimes possess widely flared lateral leaves. For each gemma, we measured the following: 1) the length of the gemma along its longest axis; 2) the width of the gemma at its widest point; 3) the length of the upper leaf and 4) the length of the lower leaf.

To evaluate the effects of drying on gemma shape and size, the length and width of 12–20 gemmae from representative collections of from two individuals belonging to three species that span most of the range of observed gemma sizes were measured prior to and after pressing and drying. These were: *H. appressa* (*Testo 345*, VT, 20 gemmae; *Corwin s.n.*, VT, 13 gemmae), *H. lucidula* (*Testo 346*, VT, 18 gemmae; *Testo 403*, VT, 14 gemmae) and *H. selago* (*Testo 330*, VT, 15 gemmae; *Testo 330A*, VT, 13 gemmae).

All measurements were obtained at 40× magnification under a Leica MZ8 stereoscope fitted with a SPOT Insight Firewire 2.0 camera (Spot Imaging Solutions, Sterling Heights, MI, USA). Angles of leaf apices were measured on

composite drawings made by stacking 8–10 images of individual gemmae from each species using Adobe Photoshop CS2 software and tracing averaged leaf outlines. Composite images of whole gemmae were obtained by stacking 15–20 images taken at 20× magnification.

Results and Discussion

Sizes.—Overall gemma size differed across species (Fig. 2). Gemma lengths (±SD) ranged from 2.54 ± 0.26mm for *H. arctica* to 4.84 ± 0.24mm for *H. lucidula*; gemma widths ranged from 2.16 ± 0.18mm for *H. appressa* to 4.34 ± 0.20mm for *H. lucidula*.

Upper and lower leaf lengths are given in the individual species description. In general, upper leaves range from 0.3× to 0.5× the length of the entire gemma, with the relatively shortest belonging to *H. lucidula* and *H. miyoshiana* and the relatively longest to *H. arctica* and *H. selago*. The lower leaf is always longer than the upper leaf, ranging from 0.4× to 0.6× as long as the gemmae, with the relatively shortest belonging to *H. lucidula*, *H. miyoshiana* and *H. occidentalis* and the relatively longest belonging to *H. selago*.

Shapes.—Gemma shapes range from narrowly elliptic (*H. appressa* and *H. porophila*) to broadly obovate and nearly circular (*H. haleakalae*, *H. arctica*). Other shapes include oblong (*H. miyoshiana*) to broadly obpyriform (*H. occidentalis*) and obcampanulate (*H. lucidula, H. selago*).

Upper leaf shapes range from narrowly lanceolate (*H. lucidula*, *H. selago*), to oblong (*H. haleakalae*) and narrowly elliptic (*H. arctica*).

Lateral leaves are distinctively shaped for some species, particularly the very broad, somewhat squarish ones of *H. lucidula* (Fig. 3). Those of *H. haleakalae* and *H. arctica* are strongly and evenly curved along their outer margins, while their inner margins are nearly straight, resulting in a nearly circular appearance of the gemma as a whole. Angles of the lateral leaf apices range from narrowly acute to slightly obtuse, with mucronulate tips being found in several species, most notably *H. lucidula* and *H. selago*.

Shapes of the central leaf vary from oblong to narrowly elliptic, with an obtuse to acute apex.

The lower leaves are mostly uniform in their relative length, but vary in shape similarly to the upper leaf, ranging from narrowly lanceolate (*H. porophila*) to oblong (*H. appressa*, *H. selago*) to slightly pandurate (*H. haleakalae*, *H. miyoshiana*).

Descriptions of gemmae of North American Huperzia.—

Huperzia appressa.—(Fig. 3A, I) **Outline** biconvex to narrowly obovate, broadest above middle at 0.75–0.85× of overall length; length [2.7] 3.0–3.4 [3.7] mm; width [1.7] 2.0–2.3 [2.5] mm. **Upper leaf** ca. 0.4×–0.45× as long as gemma, slightly pandurate, narrowing distally to an elongate, acute apex, margins meeting at ca. 50°. **Central leaf** lanceolate to oblong, with an acute apex at ca. 75°. **Lateral leaves** relatively narrow, outer margins straight from base to near middle, then curving to acute apices, margins meeting at ca. 60°.

FIG. 3. Gemmae of North American *Huperzia*. Images above species names are adaxial surface views, corresponding images below species names are abaxial surface views. White lines outline the shape of the upper and lower leaves.

Lower leaf ca. 0.5× as long as gemma, oblong with a broadly acute apex with an angle of ca. 80°–85°.

Huperzia appressa has the narrowest gemmae of all North American species, often appearing lens-shaped. The lateral leaves are relatively thin compared to those of other eastern North American species. Gemmae of this species are produced continuously during the growing season, unlike those of other species in its range, which are clustered near the tip of each year's growth.

Huperzia arctica.—(Fig. 3B, J) **Outline** broadly obovate to nearly circular, broadest above middle at 0.65–0.75× of overall length; length [2.1] 2.4–2.7 [3.0] mm; width [1.9] 2.1–2.3 [2.5] mm. **Upper leaf** ca. 0.5× as long as gemma, narrowly elliptic, apex acute, margins meeting at ca. 35°. **Central** leaf narrowly biconvex, apex acute, margins meeting at ca. 75°. **Lateral leaves** very broadly obovate, rounded towards obtuse apices, margins meeting at ca. 95°. **Lower leaf** ca. 0.6× as long as gemma, oblong to broadly spatulate with a broadly acute apex, margins meeting at ca. 85°

As in *H. appressa* and *H. haleakalae*, gemmae of *H. arctica* are often distributed along the length of the short annual shoot increments. Some authors (Tolmachev, 1960; Dignard, 2014) have noted that gemmae are particularly abundant in this taxon. The outside edges of the lateral gemmae leaves often curve toward the adaxial surface, producing a cupped appearance. Like the entire plant, the gemmae are yellow in color and lustrous.

Huperzia haleakalae.—(Fig. 3C, K) **Outline** very broadly obovate to nearly circular, broadest above the middle at 0.55–0.60× of overall length; length [2.7] 3.0–3.2 [3.4] mm; width [2.6] 2.9–3.1 [3.2] mm. **Upper leaf** ca. 0.45× as long as gemma, oblong, narrowing abruptly to apex, margins meeting at ca. 90°. **Central leaf** biconvex, narrowing to an acute tip, margins meeting at ca. 65°. **Lateral leaves** nearly semi-circular; outer margins evenly curved to apices, margins meeting at ca 90°. **Lower leaf** ca 0.50–0.60× as long as gemma, biconvex or slightly pandurate with a ± broadly spathulate tip; apex acute, margins meeting at ca. 75°.

Gemmae of *H. haleakalae* are notably rounded due to strong curvature of the lateral leaf margins; this shape and their relatively small size easily distinguish this species from *H. miyoshiana*, and *H. selago,* with which it overlaps in much of its range. They are yellow in color and lustrous in appearance, similar to those of *H. arctica*. This shared color, along with the similarity in gemma shape and their distribution all along the shoot, may imply a close relationship between these species.

Huperzia lucidula.—(Fig. 3D, L) **Outline** broadly obcampanulate, flaring to a broad distal portion, broadest above the middle at ca. 0.75–0.8× of overall length; length [4.4] 4.7–4.9 [5.4] mm; width [3.8] 4.2–4.5 [4.6]. **Upper leaf** ca. 0.25×–0.3× as long as gemma, narrowly lanceolate, narrowing to an elongate, acuminate apex, margins meeting at ca. 15°–20°. **Central leaf** broadly oblong, margins nearly parallel to near tip, then curving abruptly to a mucronulate, broadly obtuse apex, margins meeting at ca. 140°–150°. **Lateral leaves** broadly widening from bases to near distal end, apices mucronulate,

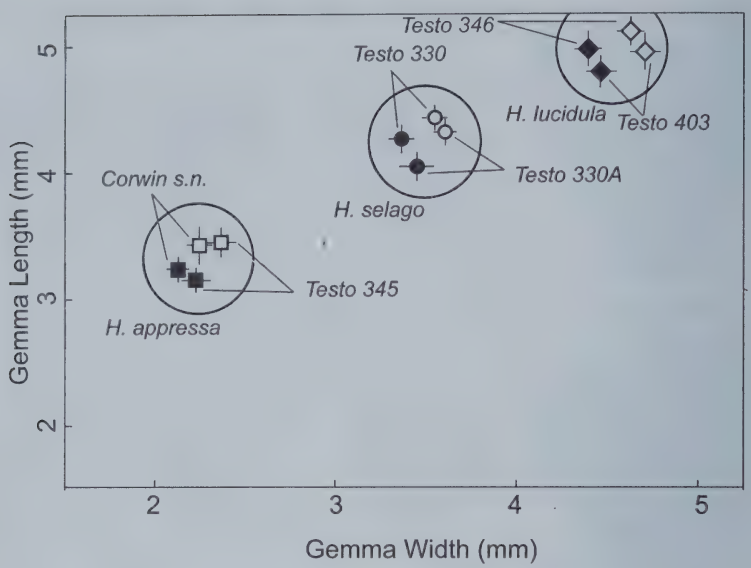

FIG. 4. Measurements of fresh and dried gemmae from *Huperzia appressa* (squares), *Huperzia lucidula* (diamonds), and *Huperzia selago* (circles). Open symbols represent fresh gemma measurements, closed symbols represent dried gemma measurements. Error bars represent ± 1 standard deviation.

very broadly obtuse, margins meeting at ca. 165°–180°. **Lower leaf** ca. 0.45× as long as gemma, narrowly oblong, slightly pandurate, apex rounded to acute.

Huperzia lucidula possesses the largest gemmae of any North American *Huperzia*. The very broad outline, mucronulate lateral leaf apices, and small, narrow upper leaf is distinctive. Its gemmae are dark green, a color shared by *H. porophila*, which is an allopolyploid thought to be derived in part from this species (Waterway, 1986; Wagner and Beitel, 1993).

Huperzia miyoshiana.—(Fig. 3E, M) **Outline** broadly oblong-elliptical, broadest above middle at 0.65–0.75× of overall length; length [3.5] 3.8–4.1 [4.3] mm; width [2.7] 2.9–3.1 [3.4] mm. **Upper leaf** ca. 0.3× as long as gemma, narrowly lenticular with an acute apex, margins meeting at ca. 60°. **Central leaf** biconvex, with an acute apex; margins meeting at ca. 70°. **Lateral leaves** broadly elliptic with acute apices, forming an angle of ca. 70°. **Lower leaf** ca. 0.45× as long as gemma, often slightly pandurate; apex broadly acute, margins meeting at ca. 80°.

Huperzia miyoshiana is easily distinguished from other species in its range by the sea-green color of its gemmae, which are produced in a series of 2–3 (rarely to 10) pseudowhorls at the summit of the year's growth and frequently give the shoot apex a 'bulbous' appearance. The lateral leaves of its gemmae are relatively thin, giving them a less fleshy appearance than those of other western North American species.

Huperzia occidentalis.—(Fig. 3F, N) **Outline** broadly obovate to obpyriform, broadest at 0.60–0.70× of overall length; length [4.0] 4.2–4.5 [4.6] mm; width

[3.1] 3.3–3.5 [3.6] mm. **Upper leaf** ca. 0.4× as long as gemma, broadly lanceolate, narrowed to an acute apex, margins meeting at ca. 65°. **Central leaf** spathulate with an acute to acuminate tip, margins meeting at ca. 80°. **Lateral leaves** with outer margins widely bowed out distally and evenly rounded to acute apices; margins meeting at ca. 85°. **Lower leaf** ca. 0.45× as long as gemma, broadly elliptic, narrowing to an acute tip, margins meeting at ca. 75°.

The narrowly elliptic lower leaf, large size, and broadly obovate to obpyriform shape of the gemma distinguish *H. occidentalis* from other species in its range. In growth habit and overall aspect this species resembles *H. lucidula* from eastern North America and has historically been treated as both a form and variety of this species. Differences in gemma size and shape and especially the shape of the upper leaf (lenticular in *H. occidentalis*, narrowly lanceolate in *H. lucidula*) support the distinctiveness of this Pacific Northwest and Rocky Mountain native.

Huperzia porophila.—(Fig. 3G, O) **Outline** narrowly elliptic to narrowly obovate, broadest above middle at 0.65–0.75× of overall length; length [3.5] 3.8–4.1 [4.2] mm; width [2.4] 2.6–2.9 [3.1] mm. **Upper leaf** ca. 0.35× as long as gemma, narrowly lanceolate, narrowly acute, margins meeting at ca. 25°. **Central** leaf narrowly oblong, acute, margins meeting at ca. 30°. **Lateral leaves** oblong-elliptic; tips acute, margins meeting at ca. 30°; inner margins (exposed above the central leaf) nearly straight. **Lower leaf** ca. 0.4× as long as gemmae, lanceolate, narrowly acute.

Gemmae of this species are intermediate in shape between those of its progenitors, *H. lucidula* and *H. appressa* (Wagner and Beitel, 1993). The oblong-elliptic lateral leaves and relatively narrow overall shape support the hypothesis of *H. appressa* parentage and the narrow lower and upper leaves and the gemma's dark green color are consistent with *H. lucidula* parentage for this species.

Huperzia selago.—(Fig. 3H, P) **Outline** narrowly obcampanulate, broadest towards the apex at 0.8× of overall length; length [3.6] 4.0–4.4 [4.6] mm; width [3.0] 3.3–3.6 [3.8] mm. **Upper leaf** ca. 0.45× as long as gemma, lanceolate; apex slightly obtuse, margins meeting at ca. 105°. **Central leaf** broadly oblong, with an obtuse, mucronulate apex; margins meeting at ca. 140°–150°. **Lateral leaves** narrowly obovate; margins slightly convex; tips obtuse to broadly acute, margins meeting at ca. 120°. **Lower leaf** ca. 0.6× as long as gemma, oblong; apex obtuse, margins meeting at ca. 95°.

Numerous infraspecific taxa and segregates of *H. selago* have been described by various authors over the years (e.g., Nessel, 1939) as taxonomists have struggled with its wide distribution and variability. Though several lines of evidence suggest that this taxon is still poorly known in North America, gemmae from specimens that we have examined — which span the known range of the species in North America and include new regional records — display a consistent and distinct morphology. The relatively long, oblong outer leaves, distally broadened lateral leaves, and bright green color are distinctive. Overall, the shape of *H. selago* gemmae is quite different from other species with which it is confused in North America, notably *H. appressa*

and *H. haleakalae*. Additionally, production of gemmae in this species is confined to one or two pseudowhorls at the summit of the year's growth, not all along the shoot as in those species.

Comparison of fresh and dried Huperzia *gemmae.*—For the three *Huperzia* species for which we compared fresh and dried gemmae, we found a consistent pattern of 5–15% reduction in both length and width (Fig. 4). Relative proportions and shapes of gemma leaves were indistinguishable between fresh and dried material for all species examined. Although we did not attempt to quantify color of fresh vs. dried gemmae, it was evident that no discoloration occurred during the drying process. Though the descriptions and key provided here are based on dried material, they can be used on fresh material if the size differences are accounted for. It should be noted that some gemmae on a plant can be disfigured during the process of pressing and drying; this is readily evident upon inspection. Like any herbarium material, gemma color can fade with age and exposure to light; such color changes are usually similar across all parts of the plant. Caution should be used when evaluating gemma color on old or otherwise discolored material.

Key to North American *Huperzia* Emphasizing Gemma Characters

Note: measurements are taken from dried specimens. Gemmae of herbarium specimens are 5–15% smaller in all dimensions than those of living plants.

1. Gemmae distributed throughout the length of the annual shoot increment (if gemmae are not present throughout, inspect for gemmiphores) . . . . . 2.
   2. Gemma 3.0–3.4 × 2.0–2.3mm; shape biconvex to obovate; color medium to dark green; plants of eastern North America . . . . . *H. appressa*
   2. Gemma 2.0–3.2 × 2.1–3.1mm; shape rounded; color yellowish and glossy; plants of northern and western North America . . . . . 3.
      3. Gemmae mostly <3.0mm long; plants mostly of alpine meadows and talus slopes . . . . . *H. haleakalae*
      3. Gemmae mostly >3.0mm long; plants of tundra . . . . . *H. arctica*
1. Gemmae distributed near the tip of the annual shoot increment in 1–3 [–4] pseudowhorls; not throughout the length of the annual shoot increment . . . . . 4.
   4. Plants of forests and shaded rock outcrops or overhangs in eastern North America; gemmae broadly obcampanulate or narrowly elliptic . . . . . 5.
      5. Gemmae 4.7–4.9 × 4.2–4.5mm; broadly obcampanulate, nearly as wide as long; upper leaf narrowly lanceolate; lateral and central leaves broadly obtuse with mucronulate tips; plants of forests . . . . . *H. lucidula*
      5. Gemmae 3.8–4.1 × 2.6–2.9mm; obovate, 1.5× long as wide; upper leaf narrowly lanceolate; lateral and central leaves acute; plants of rock outcrops and overhangs . . . . . *H. porophila*
   4. Plants of forests, wetlands, and alpine meadows in western North America or widely distributed but generally not of eastern forests or shaded rock outcrops or overhangs (except some *H. selago*); gemmae not broadly obcampanulate nor narrowly elliptic . . . . . 6.

6. Gemmae broadly obovate to rounded; lower outer leaf broadly convex; plants of shaded forests in the Pacific Northwest . . . . . *H. occidentalis*
6. Gemmae oblong or obovate; lower outer leaf not broadly convex; plants of cliffs, wet areas and alpine meadows . . . . . 7.
   7. Gemma color deep green to bright green; shape obovate; upper leaf ca. 0.45× as long as gemma; central leaf broadly strap-shaped; lower leaf oblong . . . . *H. selago*
   7. Gemma color sea-green; shape oblong to slightly oblong-obovate; upper leaf ca. 0.3× as long as gemma; central leaf broadly biconvex; lower leaf pandurate . . . . . *H. miyoshiana*

Conclusions.—The simple body plan of *Huperzia* presents fewer systematically useful morphological characters than most plant groups, a challenge that has been manifested in long-standing and largely unresolved debate regarding both species and generic boundaries (Nessel, 1939; Ollgaard, 1987, 1992; Wagner and Beitel, 1992; Haines, 2003). To distinguish species and generate phylogenetic hypotheses, taxonomists working on the group have incorporated evidence from diverse datasets, including spore morphology (Wilce, 1972; Tryon and Lugardon, 1991), spore abortion (Beitel and Mickel 1992; Wagner and Beitel 1993), gametophyte morphology (Bruce, 1976), secondary metabolite production (Pedersen and Øllgaard, 1982), and sporangium anatomy (Wilce, 1965; Øllgaard, 1975). Here, we demonstrate that the gemmae of North American *Huperzia* provide ample characters for differentiating species and suggest that the same characters will prove useful elsewhere. Phylogenetic relationships among *Huperzia* species remain too poorly understood to explore the utility of gemma characters beyond identifying species and supporting hypotheses of hybrid origin, but affinities between some species (e.g. *H. arctica* and *H. haleakalae*) are indicated by gemma morphology.

Another factor that has confounded taxonomic resolution among North American *Huperzia* is the prevalence of interspecific hybrids, which can become locally abundant as a result of asexual reproduction. Wagner and Beitel (1993) commented extensively on the challenges presented by hybrids, which they described as "extremely common" and indicated were the source of considerable confusion experienced by North American students of the genus. Though describing gemma morphology of hybrids was not the goal of our study, we did encounter a number of sterile hybrids among the specimens we examined; these plants exhibited gemma characters intermediate to those of their putative parents. Similarly, the gemmae of the allopolyploid *H. porophila* are intermediate in size and shape to *H. lucidula* and *H. appressa* (Fig. 3), supporting the hybrid origin proposed for this species by Wagner and Beitel (1993) and opposing that of Waterway (1986), who suggested involvement of *H. selago* instead of *H. appressa*. With this pattern of intermediacy in mind, we suggest that plants possessing gemmae intermediate in size and shape to the descriptions provided here should be examined for abortive spores and other signs of hybridity, such as intermediate leaf shape and stomate distribution.

Though it is not the goal of this paper to report on ranges or reach broad taxonomic conclusions for North American *Huperzia* species, and although our sample size was limited in scope, nevertheless we encountered several noteworthy specimens. First, we identified a specimen of *H. miyoshiana* (*Calder and Taylor 23305,* GH) collected at Tilt Cove, in eastern Newfoundland. This is just the second known locality for *H. miyoshiana* in eastern North America (Brunton *et al.*, 1992) and extends the species' range eastward approximately 170 km. Second, although Wagner and Beitel (1993) reported that, "Plants from Greenland formerly identified as *Huperzia selago* are *H. appalachiana*," and all *Huperzia* specimens from Greenland should be referred to *H. appalachiana* (= *H. appressa)*, we have encountered multiple specimens from southern Greenland that are clearly *H. selago*. During our studies we identified several specimens of *H. arctica*, a species not mentioned by Wagner and Beitel (1993) from northern Greenland. We also encountered specimens of *H. selago* from British Columbia, northern Labrador, and the western United States, all of which are beyond the range reported for this species by Wagner and Beitel (1993). In addition, we report two significant expansions of the known range of *H. haleakalae*, which in continental North America is known from the Pacific Northwest with several disjunct populations in central Rocky Mountain states. One specimen (*Raup 6367*, GH) was from the northern shore of Lake Athabaska in Saskatchewan; the other (*Abbe and Abbe 3171*, GH), from Richmond Gulf in northern Quebec, extends the range of *H. haleakalae* eastward by approximately 3000km. Other specimens not studied here suggest that the range of *H. haleakalae* may extend even further than our study indicates (A. Haines, personal communication) and the taxonomic status of this species (type from Hawaii) is being revisited. These new records highlight the need for further studies of gemma morphology and other characters in an effort to improve our understanding of species boundaries and distributions among North American *Huperzia*. To address these needs, we are currently working toward a revision of the genus in North America.

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APPENDIX 1. Material Examined

Huperzia appressa (Desv.) Á. Löve & D. Löve

CANADA. Ontario: Thunder Bay Dist., Near SW end of Thompson Island, *Abbe and Bierhorst 5204* (VT). S of Highway 61, 15 mi. E of Pigeon River, *Abbe and Bierhorst 5121* (VT).

U.S.A. Maine: Piscataquis Co., Mt. Katahdin, "shaded cliffs," *Fernald s.n.* (VT). Baxter State Park, Mt. Katahdin, "at timberline in moist spots," *Milstead 1094* (VT). Minnesota: Lake Co., Superior National Forest, 9.2 miles NNW of Isabella, prominent rock outcrop *Gerdes 5397* (MN). Superior National Forest, 9.2 miles NNW of Isabella, *Gerdes 5489* (MN). Superior National Forest, 9.2 miles NNW of Isabella, *Gerdes 5505* (MN). Superior National Forest, 9.2 miles NNW of Isabella, *Gerdes 5506* (MN). New Hampshire: Coos Co., Mt. Washington, *Flynn s.n.* (VT). Lion's Head Trail, Mt. Washington, *Hill 624* (VT). Grafton Co., Franconia, summit of Mt. Lafayette, *Anon. s.n.* (VT). New York: Essex Co., Northern sector of plateau of Mt. Skylight, *Pruski 3550* (NY). Franklin Co., Algonquin Mountain, *Cook 738* (VT). North Carolina: Mitchell Co., Roan Mountain, *Vasey s.n.* (VT). Yancey Co., summit of Mt. Mitchell, *Hill 38364* (VT). Vermont: Chittenden Co., Underhill, Mt. Mansfield, *Zika 4722* (VT). Underhill, summit of Mt. Mansfield, *Testo 345* (VT). Mt. Mansfield, *Pringle s.n.* (VT). Lamoille Co., Cambridge, Smuggler's Notch, *Zika 4749* (VT). Orleans Co., Lowell, Haystack Mountain, *Charette 2233* (VT). Washington Co., Duxbury, summit of Camel's Hump, *Blanchard s.n.* (VT).

Huperzia arctica (Gross. *ex* Tolm.) Sipliv.

CANADA. Ontario: Kenora Dist., Winisk, Hudson Bay Lowlands, west of James Bay, *Lundsden s.n.* (NY). Nunavut: Baffin Reg., Frobisher Bay, Baffin Island, *Senn and Calder 4032* (GH, VT). Baffin Island, Southeast Cape Hooper, Tanner Bay, *Elven 3503/99* (ALA). Keewatin Reg., Cape Fullerton, Hudson's Bay, *Macoun s.n.* (GH). Kitikmeot Reg., Ukkusiksalit Nat'l. Park, Walker Bay, *Tremblay 85-2005* (GH).

GREENLAND. Qaasuitsup, Disko Island, Lyngmarken, *Pedersen s.n.* (GH). Cape York, *Wetherill 50* (GH). Disko Island, Godhavn, *Krumlein s.n.* (GH). Disko Island, *Porsild s.n.* (GH). Harvard Island, Inglefield Gulf, *Angel 36*, (NY). Egedesminde, *Rink s.n.* (GH). Grenville Bay, Region of North Star Bay, *Ekblaw 29* (GH). Northeast Greenland Nat'l. Park, W coast of Cape Hedlund, Kempe Fjord, *Seidenfaden 472* (GH). Near Mestersvig, Kong Oscar's Fjord, *Raup 222* (GH). Sermersoog, Liverpool Land, S side of Hurry Island, Kalkdel Inlet, *Sørensen s.n.* (GH).

U.S.A. Alaska: Arctic Reg., Kotzebue, Kotzebue Sound, *Scamman 3950* (GH). Far North Reg., Flaxman Island, near Bullen Point, *Keller* 1232 (ALA). West side of Jago River, *Cantlon and Gillis 57-626* (ALA). Bering Strait District, Cape Dyer, drainage of Kipaloq and Angowlik creeks, *Viereck 4100* (ALA). Philip Smith Mountains, Uyamitquaq Creek and vicinity, *Batten 85-337* (ALA). Southcentral Reg., 13 mi. west of Paxson on Denali Road, *Harms 4192* (ALA).

Huperzia haleakalae (Brack.) Holub

CANADA. British Columbia: Columbia-Shuswap Dist., Glacier Nat'l. Park, Asulkan Valley, *Brown 585a* (GH; mixed sheet with *H. occidentalis, Brown 585b*). Northern Rockies Dist., Below Mt. St. George, Mile 393 Alaska Hwy., *Calder and Gillett 26592* (WTU). Quebec: Nunavik, Richmond Gulf, S of Cairn Island, "moist pockets and crack in granite hills," *Abbe and Abbe 3171* (GH). Saskatchewan: Northern Reg., Vicinity of Charlot Pt., Lake Athabaska, *Raup 6367* (GH; mixed sheet with *H. selago, Raup and Abbe 4591*). Yukon: North Yukon, Tombstone Nat'l. Park, Ogilvie Mountains, mountain E of mi. 50-54, *Porsild 175* (WTU). Ogilvie Mountains, North Fork Pass, *Parker 1199* (ALA).

U.S.A. Alaska: Arctic Reg., Seward Peninsula, Nome, *Neville Jones 8967* (WTU; mixed sheet with *H. selago, Thompson 14443*). Far North Reg., Waring Mountains, vicinity 3km west of VABM Slam, *Parker 9340* (ALA). Survey Pass, vicinity of Altna and Nahtuk rivers, *Murray 3852* (ALA). Interior Reg., Along Porcupine River, *Turner s.n.* (VT). NE of Cripple Creek, Steese Hwy. NE of Fairbanks, *Gilman 99220* (VT). Denali National Park and Preserve, NW slope to summit of Mt. Eielson, *Viereck 1452* (GH). Southcentral Reg., Matanuska-Susitna Borough, Hatcher Pass Rd., *Goldman 3465* (VT). Vicinity of Schwan Glacier terminus, *Parker 1931* (ALA). Wrangell-St. Elias Park & Preserve, Euchre Mountain, *Bennett and Loomis 03-806* (ALA). Southwestern Reg., Southeastern Kagalaska Island, "steep open slope, near and above pond shore," *Zika 16970* (VT, WTU). Saint Matthew Island, *Ward and Ward 26* (ALA). Bering Sea Region, Saint Matthew Island, *Murray 12531* (ALA). Nulato Hills, 25km southeast of Unalakleet, *Parker 7003* (ALA). Washington: Chelan Co., North Cascades, 3 air km N of Blue Lake, between Rainy Pass and Washington Pass, *Zika 24646* (VT, WTU). Clallam Co., Olympic Mountains, "meadows," *Piper 2232* (GH). Skagit Co., North Cascades Nat'l. Park, 1 air km S of Easy Pass, *Zika 18862* (WTU).

Huperzia lucidula (Michx.) Trev.

CANADA. Quebec: L'Estrie Reg., Comté de Compton, tourbiere de Johnville, *Guevremont 236* (VT).

U.S.A. Connecticut: Hartford Co., Farmington, Rattlesnake Mountain, *Hill 19779* (VT). Tolland Co., Mansfield, W of Mansfield City and Crane Hill Rds., *Pfeiffer 1285* (VT). Illinois: Winnebago Co., Near Fountaindale, *Bebb s.n.* (VT). Maine: Aroostook Co., Perham, Hanford Siding, *Seymour 23220* (VT). Maryland: Garrett Co., Grantsville, Crab Run Road, NW of intersection with River Road, *Hill 9539* (VT). Michigan: Houghton Co., T54N, R33W, Sec. 6, *Parmelee 2028* (VT). Minnesota: Cook Co., NE of Lutsen, "base of steep igneous rock cliff," *Brooks 2963* (VT). Superior National Forest, south of the Royal River and 0.32 miles SE of John Lake, *Gerdes 5944* (MN). Lake Co., Superior National Forest, approximately 5.74 miles NNW of Ely, *Gerdes 5707* (MN). Superior National Forest, approximately 9.6 miles north of Isabella, *Gerdes 6460* (MN). Superior National Forest, approximately 20.73 miles NW of Isabella, *Gerdes 6381* (MN). New York: Tompkins Co., Danby, Lake Cayuga, Michigan Hollow

Swamp, *Schuster s.n.* (VT). Pennsylvania: Indiana Co., White's Woods, 1 mi. NW of Indiana, *Chiesa s.n.* (VT). Vermont: Caledonia Co., Peacham, *Gilman 93001* (VT). Chittenden Co., Underhill, Mt. Mansfield, Sunset Ridge Trail, *Testo 346* (VT). Franklin Co., Fletcher, woods near Metcalf Pond, *Countryman 1133* (VT). Lamoille Co., Cambridge, E side of Smuggler's Notch, Mt. Mansfield, *Zika 4149* (VT). Cambridge, E of VT State Rt. 108, *Charette 2837* (VT). Rutland Co., Mount Tabor, Long Trail, *Seymour 24418* (VT). Tinmouth, *Carpenter s.n.* (VT). Washington Co., Worcester, moist woods, Worcester Pond, *Seymour 27729* (VT).

Huperzia miyoshiana (Makino) Ching

CANADA. British Columbia: Columbia-Shuswap Dist., Victor Lake Provincial Park, ca. 11 mi. W of Revelstoke, *Hitchcock and Martin 7574* (WTU). Queen Charlotte Islands, W coast of Graham Island, mountain near head of Shields Bay, *Calder and Taylor 23305* (GH). Newfoundland and Labrador: East Reg., North shores of Notre Dame Bay, Tilt Cove, "turfy, gravelly and ledgy crests, Castle Rock," *Fernald and Wiegand 4350* (GH).

U.S.A. Alaska: Southcentral Reg., Eleanor Island, Prince William Sound, east side of Northwest Bay, *Lewis s.n.* (ALA). Southeastern Reg., Juneau, *Scamman 1089* (GH). Near Juneau, Windfall Lake, *Anderson 6157* (GH). Prince of Wales Island, Hollis vicinity, Harris River, *Little 306* (VT). Tongass Nat'l. Forest, NE side of Port Armstrong, Baranoff Island, *Muller 4624* (WTU). Dall Island, Thunder Mountain, *Muller 5517* (ALA). Coronation Island, Egg Harbor, *Neiland 1133* (ALA). Wrangell-St. Elias National Park & Preserve, Black Glacier Creek, *Loomis 1558* (ALA). Southwestern Reg., SE Kagalaska Island, "steep open slope, near and above pond shore," *Zika 16970* (VT, WTU). Washington: Snohomish Co., Cascade Mountains, Martin Creek Trail, *Legler 68* (WTU). Mount Dickerman, *Thompson s.n.* (GH). Perry Creek trail, near Big Four Inn, *Thompson 14529* (NY).

Huperzia occidentalis (Clute) Kartesz & Gandhi

CANADA. British Columbia: Columbia-Shuswap Dist.: Glacier Nat'l. Park, Asulkan Valley, *Brown 585b* (GH; mixed sheet with *H. haleakalae, Brown 585a*).

U.S.A. Alaska: Southeastern Reg., Baranof Island, southeast end near Port Alexander, *Muller 5386* (ALA). Washington: Jefferson Co., Olympic Nat'l. Forest, 2km from trailhead to Lena Lake, *Testo s.n.* (VT). King Co., Near Snoqualmie Pass, "open rocky talus slopes," *Thompson 8918* (WTU). Pend Oreille Co., Near Roosevelt Grove of Ancient Cedars, above Granite Falls, *Zika 25077* (WTU). Snohomish Co., Cascade Mountains, Martin Creek Trail, *Legler 69* (WTU). Stevens Co.: Deer Creek, E of Kettle River, *Gilman 96189* (VT). Whatcom Co., 2 air mi. E of Beebe Mountain, Route 20 at East Creek trailhead, *Zika 25739* (WTU). Mt. Baker – Snoqualmie Nat'l. Forest, 1 air mi. NNE of Lake Wiseman, Elbow Lake Trail, *Zika 18978* (VT, WTU).

Huperzia porophila (F. E. Lloyd & Underw.) Holub

U.S.A. Iowa: Allamakee Co. Franklin, 96N 5W, Sect. 15, *Hartley s.n.* (GH). Indiana: Crawford Co., 1 mi. East of Taswell, *Deam 22376* (GH). 3 mi. W of

Fredonia, S of State Hwy., *Deam 44615* (GH). Minnesota: Cook Co., Superior National Forest, Royal River Corridor, approximately 0.07 miles south of the Boundary Waters Canoe Area Wilderness, *Gerdes 5924* (MN). Superior National Forest, Royal River Corridor, approximately 0.07 miles south of the Boundary Waters Canoe Area Wilderness, *Gerdes 5925* (MN). Boundary Waters Canoe Area Wilderness, rugged forested terrain northeast of Brule Lake, *Lee 5141* (MN). Lake Co., Superior National Forest, 9.27 miles NNW of Isabella, *Gerdes 5395* (MN). Superior National Forest, 9.27 miles NNW of Isabella, *Gerdes 5396* (MN). Missouri: Madison Co., Mine La Motte, *Palmer 31591* (GH). Ohio: Fairfield Co., Near Sugar Grove, *Jennings and Kellerman s.n.* (GH). Hocking Co., Near Logan, Hocking Hills State Park, sandstone cliff, *Testo s.n.* (VT). Unknown locality: *Dutton s.n.* (VT). Tennessee: Morgan Co., Rugby, *Svenson s.n.* (GH). Virginia: Lee Co., Vicinity of Rose Hill, sandstone cliffs, *Carr 890* (GH).West Virginia: Upshur Co., Near Carter, Natural Bridge of Laurel Fork, *Goodwin s.n.* (GH).

Huperzia selago (L.) Bernh. ex Schrank & Mart.

CANADA. Alberta: Peace River Dist., North shore of Lake Athabaska, Sand Point, *Raup and Abbe 4591* (GH; mixed sheet with *H. haleakalae, Raup 6367*). British Columbia: Kootenay Dist., Kokanee Glacier Provincial Park, "moist slopes," *Thompson 14443* (WTU; mixed sheet with *H. haleakalae, Neville Jones 8967).* Newfoundland and Labrador: Labrador, N. Labrador, Ungava Bay, *Turner 663* (GH). E. Labrador, Battle Island, Battle Harbour, *Potter and Brierly s.n.* (GH). W. Labrador, "Betechewan," *Abbe s.n.*(GH). Nova Scotia: Victoria Co., 3 mi. W of Little River, shaded ledge, *Smith 22105* (VT). Nunavut: Kitikmeot Reg., Coppermine, "on rock ledges," *Oldenburg 43-617* (GH). Quebec: Gaspé Co., River St. Anne Des Monts, calcareous cliffs, *Fernald and Collins 152* (NY, VT).

GREENLAND. Qaasuitsup: Disko Island, ca. Neria, *Eugenius s.n.* (GH). Egedesminde, *Simmons 108* (GH).

U.S.A Alaska: Far North Reg., Schwatka Mountains, Reed Hot Springs, Reed River Vally, *Parker 13820* (ALA). Southeastern Reg., Ketchikan area, above town on Deer Mountain, *Neiland 791* (ALA). Annette Island, near top of Yellow Hill, *Stensvold 7918* (ALA). Minnesota: Cook Co., Boundary Waters Canoe Area Wilderness, 24 miles northwest of Grand Marais, *Lee 5310* (MN). Lake Co., Superior National Forest, 9.2 miles NNW of Isabella, *Gerdes 5508* (MN). Spuerior National Forest, approximately 10 miles east of Babbitt, *Gerdes 5359* (MN). St. Louis Co., Superior National Forest, Steep Lake, approximately 12.36 miles ENE of Crane Lake, *Gerdes 6939* (MN). Montana: Flathead Co., Glacier Nat'l. Park, 1 mi. S of Akokala Lake, Numa Ridge Fen, *Lesica 11044* (WTU). Ravalli Co., Along streamlet in deep cut, between Sheafman Pass and Fred Burr Creek, *Lackschewitz 2404* (NY). New Hampshire: Grafton Co., Beaver Brook, Mt. Moosilauke, *Copeland s.n.* (NY). New York: Essex Co., On rock in partial shade, Avalanche Lake, *Copeland 1412* (NY). Vermont: Caledonia Co., Peacham, S side of Peacham Pond, *Gilman s.n.* (VT). Lamoille Co., Cambridge, Smuggler's Notch, *Jones s.n.* (VT). Eden, Belvidere Mountain, old asbestos mine pit, *Testo 330* (VT). Johnson, *Grout s.n.* (VT).

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A Decade of New Pteridophyte Records for the State of Veracruz, Mexico

Thorsten Krömer*, César I. Carvajal-Hernández, and Amparo R. Acebey
Centro de Investigaciones Tropicales, Universidad Veracruzana, Casco de la Ex Hacienda Lucas Martín, Privada de Araucarias S/N. Col. Periodistas, C.P. 91019, Xalapa, Veracruz, Mexico, *e-mail: tkromer@uv.mx (corresponding author)

Alan R. Smith
University Herbarium, 1001 Valley Life Sciences Bldg. #2465, University of California, Berkeley, CA 94720-2465, USA, e-mail: arsmith@berkeley.edu

Abstract.—Veracruz, with 542 recorded species, is the third richest state in Mexico in terms of total fern diversity. Field work, herbarium studies, and a revision of literature during the last decade revealed 22 new state records. Five of these belong to *Elaphoglossum*, four are filmy ferns, and three are grammitids. Most of the new taxa were collected in the endangered humid montane and pine-oak forests of the central and Los Tuxtlas area during recent research projects. Another 13 species, mainly within *Elaphoglossum* and *Selaginella*, have been published as new records in the literature. Whereas several of these might possibly be confirmed in the future, others form part of poorly known species complexes that require specific studies, and we consider them as doubtful or unverified. Our study highlights the need of more fern inventories in remote and mostly unexplored areas, as well as revisions of national and local herbaria, both of which may reveal additional species new to science or range extensions.

Key Words.—*Elaphoglossum*, ferns, filmy ferns, grammitid ferns, lycophytes, *Selaginella*

The publication of *The Pteridophytes of Mexico* by Mickel and Smith (2004) confirmed that Mexico has one of the most diverse fern floras of the world. It harbors approximately 124 genera and 1008 species, of which 186 are endemic to the country. The state of Veracruz, with 542 recorded species, is the third richest in terms of total fern diversity, after Chiapas (697 species) and Oaxaca (667) (Mickel and Smith, 2004). However, herbarium revisions and field work, particularly in montane areas of difficult access, may still reveal species new to science or range extensions, even in floristically well-known states as Veracruz with an estimated 7800 species of vascular plants (Castillo-Campos *et al.*, 2011).

The availability of the Mickel and Smith treatment facilitated the identification of fern collections in floristic inventories and also furthered studies on pteridophytes and vascular epiphytes in local or regional areas. Thus, especially in Veracruz, many recent collections have been made and cited in mostly unpublished student theses or other research projects (e.g., Carreño-Rocabado, 2006; Carvajal-Hernández, 2011; Gómez-Díaz, 2010; Hernández-Rojas, 2010; Krömer *et al.*, 2013a; Mehltreter, 2008; Viccon-Esquivel, 2009). These works have considerably increased our knowledge of the distribution of ferns in the state. In addition, these studies revealed one fern species new to science and several new records for Mexico and Veracruz

FIG. 1. Map of Veracruz, Mexico, and bordering states. Darker shades of gray indicate increasing elevation.

(Acebey and Krömer, 2010; Acebey *et al.*, 2015; Carvajal-Hernández *et al.*, 2014; Carvajal-Hernández and Krömer, in press; Krömer *et al.*, 2007, 2013b).

Additional fern records for Veracruz were mentioned in Tejero-Díez *et al.* (2011), but these were based mainly on data from Palacios-Ríos (1992) and Rojas-Alvarado (2003); however, it has not always been possible in our study to find and verify the cited vouchers. Although these reports have increased significantly the total of fern species for Veracruz, the information is dispersed or not widely available; moreover, there are still unpublished range extensions. The purpose of our work was to compile an annotated list of new fern records for Veracruz State. The treatment of Mickel and Smith (2004) was used as the basis to determine whether a species is a new record for the state.

MATERIALS AND METHODS

Extensive field work, mainly in the central montane region of Veracruz and the Los Tuxtlas area (Fig. 1), resulted in more than 1250 fern collections. These have been deposited at the following herbaria: Instituto de Investigaciones Biológicas, Universidad Veracruzana, Xalapa (CIB), Herbario Nacional de México, Universidad Nacional Autónoma de México, Distrito Federal (MEXU),

Marie Selby Botanical Gardens, Sarasota, Florida (SEL), División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana Iztapalapa, Distrito Federal (UAMIZ), University Herbarium, University of California, Berkeley (UC) and/or Instituto de Ecología, A. C., Xalapa (XAL). The field studies were conducted between 2005 and 2014 by the authors and/or collaborators in the course of Masters, Doctoral, and Postdoctoral studies on the diversity and distribution of ferns and vascular epiphytes. Furthermore, we conducted revisions of herbarium specimens and made high-resolution photos of ferns from Veracruz deposited in the following herbaria: CIB; Facultad de Ciencias Biológicas y Agropecuarias, Universidad Veracruzana, Córdoba (CORU); Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Distrito Federal (ENCB); MEXU; SEL; UAMIZ; UC; and XAL.

The list below of new fern records for Veracruz follows the taxonomic order of Mickel and Smith (2004); however, recent nomenclatural changes of several species are mentioned, and information about distribution at municipality level (one voucher specimen is cited), as well as data on the life form, vegetation type, and elevational range for species in Veracruz are provided. The relevant distribution data on global and Mexican state levels for each species can be found in Mickel and Smith (2004).

Results

Our revision revealed 22 species in 16 genera as new records for the state of Veracruz. Furthermore, 13 species in seven genera are considered as doubtful or unverified reports:

1. *Argyrochosma incana* (C. Presl) Windham

Representative specimen from Veracruz: Perote: *G. Castillo C. et al. 16418* (MEXU).

Ecology: Saxicolous and terrestrial on rocky slopes; in central Veracruz in arid scrubland at 2500 m.

Note: This species was published as a new state record for Veracruz in Palacios-Ríos (1992) and Tejero-Díez *et al.* (2011); however, the cited voucher specimen in Palacios-Ríos (*R. Ortega & Pattison 2183*, XAL) has not been found. Because *A. incana* in Mexico is widely distributed and has been reported in Mickel and Smith (2004) from six neighboring states, its presence in Veracruz was expected.

2. *Astrolepis integerrima* (Hook.) D. M. Benham & Windham

Representative specimen from Veracruz: Perote: *L. Cruz P. et al. 183* (CIB, MEXU, UC, XAL).

Ecology: Saxicolous and terrestrial on rocky slopes; in central Veracruz in arid scrubland at 2750 m.

Note: Because *A. integerrima* in Mexico is widely distributed and has been reported in Mickel and Smith (2004) from six neighboring states, its presence in Veracruz was expected.

3. Asplenium peruvianum Desv.

Representative specimens from Veracruz: Calcahualco: *T. Krömer et al. 3822* (MEXU, UC).

Ecology: Saxicolous and terrestrial in shaded rock crevices; in central Veracruz in pine forests at 3100 m.

Note: This species was published as new state and country record for Veracruz and Mexico, in Acebey and Krömer (2010). It is known from only a single collection in Mexico, and thus we consider it extremely rare. In South America it is distributed widely from Venezuela to Chile.

4. Bolbitis serratifolia (Mert. ex Kaulf.) Schott

Representative specimen from Veracruz: Las Choapas: *A. Mendoza R. et al. 921* (UAMIZ).

Ecology: Terrestrial; in Uxpanapa area in tropical lowland rain forests at 30 m.

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011); it is known from only a single collection.

5. Cheilanthes complanata var. *complanata* A. R. Sm. [= *Gaga complanata* (A. R. Sm.) Fay W. Li & Windham (see Li *et al.*, 2012)].

Representative specimen from Veracruz: Huayacocotla: *J. Viccon E. 218* (CIB, MEXU, UC).

Ecology: Saxicolous on rocky slopes; in northern Veracruz in humid montane forests at 1500 m.

Note: This species has not been published as new state record for Veracruz until now; it is known from only a single collection.

6. Diplazium grandifolium (Sw.) Sw.

Representative specimen from Veracruz: Las Choapas: *A. Mendoza R. et al. 926* (UAMIZ).

Ecology: Terrestrial; in Uxpanapa area in tropical lowland rain forests at 30 m.

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011). It is known from only one collection.

7. Elaphoglossum glabellum J. Sm.

Representative specimen from Veracruz: Mecayapan: *T. Krömer & E. Otto 2871* (MEXU, UC).

Ecology: Epiphytic; in Los Tuxtlas area in pine-oak forests of the Santa Marta volcano at 850 m.

Note: This species was published as new state record for Veracruz in Acebey *et al.* (2015); it is known from only a single collection.

8. *Elaphoglossum pringlei* (Davenp.) C. Chr.

Representative specimens from Veracruz: Acajete: *C. I. Carvajal H. et al. 675* (CIB, MEXU, UC); Banderilla: *T. Krömer et al. 3738* (CIB, MEXU, UAMIZ, UC); Chiconquiaco: *C. I Carvajal H. & S. Armenta M. 406* (CIB, MEXU, UC);

Mecayapan: *T. Krömer & E. Otto 2872* (MEXU, UC); Tlacolulan: *C. I Carvajal H. & S. Armenta M. 291* (CIB).

Ecology: Epiphytic; in central Veracruz and Los Tuxtlas area in humid montane and pine-oak forests at 850–2250 m.

Note: This species was published as new state record for Veracruz in Acebey *et al.* (2015), but has also been recorded in Carvajal-Hernández and Krömer (in press). Mickel and Smith (2004) mentioned that the species is represented by many collections in Oaxaca and none from other states; however, as we record *E. pringlei* from five different locations, we also consider it common in Veracruz.

9. Elaphoglossum rufescens (Liebm.) T. Moore

Representative specimens from Veracruz: Tlacolulan: *D. Jimeno S. 1097* (CIB, MEXU, UC).

Ecology: Saxicolous on rocks; in central Veracruz in pine-oak forest at 2400 m.

Note: This species has not been published as new state record for Veracruz until now; it is known from only a single collection.

10. Elaphoglossum squarrosum (Klotzsch) T. Moore

Representative specimens from Veracruz: Acajete: *C. I. Carvajal H. & S. Armenta M. 385* (CIB, MEXU, UC).

Ecology: Saxicolous on rocks; in central Veracruz in pine-oak forests at 2500 m.

Note: This species was published as new state record for Veracruz in Carvajal-Hernández and Krömer (in press); it is known from only a single collection.

11. Elaphoglossum tambillense (Hook.) T. Moore

Representative specimens from Veracruz: Acajete: *C. I. Carvajal H. & S. Armenta M. 387* (CIB, MEXU, UC).

Ecology: Epiphytic; in central Veracruz in pine-oak forests at 2500 m.

Note: This species was published under *E. pallidum* (Baker ex Jenman) C. Chr. as a new state record for Veracruz in Carvajal-Hernández and Krömer (in press); however, although recognized in Mickel and Smith (2004), this species is now considered (by F. Matos, pers. comm.) a synonym of *E. tambillense*, as previously treated in Mickel and Beitel (1988). It is known from only a single collection from Veracruz.

12. Hymenophyllum fragile (Hedw.) C. V. Morton

Representative specimen from Veracruz: Orizaba?: *C. G. Pringle 6079* (UC).

Ecology: Epiphytic or saxicolous; in central Veracruz in humid montane forests at 1460 m.

Note: This species was previously recorded for Veracruz by Pacheco and Riba (1991) on the basis of *Conant et al. 825* (GH) and *Pringle 6079* (BR, E, GH); however, the duplicates of the latter voucher at NY and US were cited in Mickel and Smith (2004) as *H. tegularis* (Desv.) Proctor & Lourteig, although the same collection at UC was clearly identified by ARS as *H. fragile*.

13. Hymenophyllum lanatum Fée

Representative specimen from Veracruz: San Andrés Tuxtla: *T. Krömer & A. Acebey 2588* (MEXU, SEL, UC).

Ecology: Epiphytic; in Los Tuxtlas area in lower humid montane forests at San Martín Tuxtlas volcano between 740–1010 m.

Note: This species was published as new state record for Veracruz by Krömer *et al.* (2007) and considered in Acebey *et al.* (2015) as rare, because it is known from only three collections from the same area.

14. Lellingeria limula (Christ) A. R. Sm. & R. C. Moran (= *Stenogrammitis limula* (Christ) Labiak; see Labiak, 2011).

Representative specimen from Veracruz: Pajapan: *T. Krömer & E. Otto 2957* (MEXU, UC).

Ecology: Epiphytic; in Los Tuxtlas area in humid montane forest at the summit of San Martín Pajapan volcano at 1125 m.

Note: This species was published as new state record for Veracruz in Krömer *et al.* (2013b); it is known from only a single collection and thus has been classified as Critically Endangered (CR), considering the International Union for the Conservation of Nature and Natural Resources Red List Criteria at Regional Levels (IUCN, 2003).

15. Melpomene peruviana (Desvaux) A. R. Sm. & R. C. Moran

Representative specimen from Veracruz: Perote: *J. H. Beaman 2173* (MEXU, UC, US).

Ecology: Saxicolous in shaded rock crevices and on cliffs; in central Veracruz in alpine vegetation at 3930 m.

Note: This species was published as new state record in Krömer *et al.* (2013b), where it is known from only a single collection and thus has been classified as Critically Endangered (CR) considering the IUCN (2003) Red List Criteria at Regional Levels. The duplicate of the Beaman specimen at MEXU was identified in 1994 by ARS as *M. pilosissima* (M. Martens & Galeotti) A. R. Sm. & R. C. Moran and cited in Mickel and Smith (2004) as voucher for Veracruz. However, the revision of that genus for the Flora Neotropica (Lehnert, 2013) treated this specimen as *M. peruviana*, which thus is the first record for Veracruz. *Melpomene zempoaltepetlensis* (Mickel & Beitel) A.R. Sm., as treated by Mickel and Smith (2004), is a heterotypic synonym of *M. peruviana* according to Lehnert (2013).

16. Polypodium hispidulum Bartlett

Representative specimen from Veracruz: Soteapan: *R. Riba et al. 1148a* (UAMIZ).

Ecology: Epipytic; in Los Tuxtlas area in tropical lowland rain forests of Sierra Santa Marta between 300–600 m.

Note: This species was published as new state record for Veracruz in Palacios-Rios (1992), on the basis of *Riba et al. 1148a*; the second voucher cited by Palacios-Rios (*F. Ramírez R. 514*; XAL) has not been found. The additional

vouchers, *P. E. Valdivia 1324* (XAL) and *L. Zelaya 77* (UAMIZ), mentioned in Tejero-Diéz (2005) could not be verified.

17. Polystichum muricatum (L.) Fée

Representative specimen from Veracruz: Acajete: *C. I. Carvajal H. & J. Gómez D. 344* (CIB, MEXU, UC).

Ecology: Terrestrial; in central Veracruz in pine-oak forests at 2500 m.

Note: This species was published as new state record for Veracruz in Carvajal-Hernández and Krömer (in press); it is known from only a single collection.

18. Selaginella guatemalensis Baker

Representative specimen from Veracruz: San Andrés Tuxtla: *T. Krömer & A. Acebey 2277* (MEXU, UC).

Ecology: Terrestrial; in Los Tuxtlas area in tropical lowland rain forests at 510 m.

Note: This species was published as new state record for Veracruz in Krömer *et al.* (2007) and considered in Acebey *et al.* (2015) as very rare, because it is known from only a single collection.

19. Terpsichore cultrata (Willd.) A.R. Sm. (= *Alansmia cultrata* (Willd.) Moguel & M. Kessler; see Kessler *et al.*, 2011)

Representative specimens from Veracruz: Acajete: *C. I Carvajal H. et al.* 951 (CIB); Coatepec: *A. C. Hernández R. 287* (XAL). Tlalnelhuayocan: *C. I. Carvajal H. et al. 326* (XAL).

Ecology: Epiphytic (often on tree ferns); in central Veracruz in humid montane forests between 1660–2000 m.

Note: This species was published as new state record in Krömer *et al.* (2013b); it was known from only two collections and thus classified as Endangered (EN) considering the IUCN (2003) Red List Criteria at Regional Levels. A third collection was made recently and increases slightly the distribution in central Veracruz.

20. Thelypteris tuxtlensis T. Krömer, A. Acebey & A. R. Sm.

Representative specimens from Veracruz: San Andrés Tuxtla: *T. Krömer & A. Acebey 2475* (Holotype: MEXU; Isotypes: UC, XAL).

Ecology: Terrestrial; in Los Tuxtlas area in (lower) humid montane forests of the San Martín Tuxtla volcano between 920–1360 m.

Note: This species was published as new in Krömer *et al.* (2007). It is endemic to the Los Tuxtlas area, and thus classified as In Danger of Extinction by Tejero-Díez *et al.* (2011); however, it is locally common.

21. Trichomanes hymenoides Hedw. (= *Didymoglossum hymenoides* (Hedw.) Copel. (see Ebihara *et al.*, 2006)

Representative specimens from Veracruz: Mecayapan: *T. Krömer & E. Otto 2932* (MEXU, UC); Tlacoltepec de Mejía: *C. Purpus 2929* (F, UC, US).

Ecology: Epiphytic; in central Veracruz and Los Tuxtlas area in humid montane forests between 900–1350 m.

Note: This species was previously recorded for Veracruz by Pacheco and Riba (1991) and Palacios-Ríos (1992); however, besides of the Purpus collection, they cited several specimens, which are mostly either *Trichomanes reptans* Swartz [= *Didymoglossum reptans* (Sw.) C. Presl] (e.g., *C. G. Pringle 10809*, F, UC, US) or *Trichomanes krausii* Hook. & Grev. [= *Didymoglossum krausii* (Hook. & Grev.) C. Presl] (e.g., *R. Lira 199*, ENCB, UAMIZ). A molecular study including all three widespread species is necessary to understand this taxonomically complex group.

22. Trichomanes ovale (E. Fourn.) Wess. Boer (= *Didymoglossum ovale* E. Fourn. (see Ebihara *et al.*, 2006)

Representative specimens from Veracruz: San Andrés Tuxtla: *T. Krömer & A. Acebey 2736* (MEXU, UC).

Ecology: Epiphytic; in Los Tuxtlas area in tropical lowland rain forests at 200 m.

Note: This species was published as new state record for Veracruz in Krömer *et al.* (2007) and considered in Acebey *et al.* (2015) as very rare, because it is known from only a single collection.

Doubtful or Unverified Reports

1. Anemia speciosa C. Presl

Note: This species was published as new state record for Veracruz in Palacios-Ríos (1992) and Tejero-Díez *et al.* (2011) on the basis of *M. Nee 22363* (F, MEXU, XAL), which has been seen at MEXU; however, it is misidentified, likely *A. mexicana* var. *mexicana* Klotzsch, a species with a wide distribution in Mexico and previously reported for Veracruz (Mickel and Smith, 2004). *Anemia speciosa* has been reported from limestone rocks in lowland forest of two neighboring states (Mickel and Smith, 2004), and thus its presence in Veracruz, especially in similar habitat conditions at the central Veracruz and Uxpanapa regions, is possible.

2. Campyloneurum repens (Aubl.) C. Presl

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011) and had been recorded previously by Palacios-Ríos (1992). Duplicates of the cited vouchers at NY (*T. Wendt 2820, 3471*; CHAPA, MEXU, NY) were identified by Mickel and Smith as *Campyloneurum serpentinum* (Christ) Ching, a species already recorded for Veracruz in Mickel and Smith (2004). *Campyloneurum repens* has been reported from tropical lowland rain forest of Chiapas (Mickel and Smith, 2004), so its presence in Veracruz, especially in similar habitat conditions at the Uxpanapa region, is possible.

3. Cheilanthes cuneata Kaulf. ex Link (= *Gaga cuneata* (Link) Fay W. Li & Windham; see: Li *et al.*, 2012)

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011) but has previously been recorded by Palacios-Ríos (1992). The cited voucher (*M. G. Zolá B. 851*, MEXU, XAL) has not been found. As *C. cuneata* in Mexico has been reported from shaded, often rocky slopes between 1050–2800 m in three neighboring states (Mickel and Smith, 2004), its presence in similar habitats of central Veracruz might be possible.

4. Elaphoglossum ellipticifolium A. Rojas

Note: This species was recorded for Veracruz in Rojas-Alvarado (2003) and Tejero-Díez *et al.* (2011); the cited voucher (*M. Nee et al. 26368*, F) has not been verified, whereas many other of the mentioned vouchers from Chiapas and Oaxaca (e.g., *D. Breedlove 15292, 23036*, NY; *J. Mickel & R. Hellwig 4124*, UC, US) are cited in Mickel and Smith (2004) under *E. leebrowniae* Mickel, which thus might be a synonym of *E. ellipticifolium* as indicated by Tejero-Díez *et al.* (2011). Because *E. leebrowniae* has been reported in Mexico from wet pine-oak forests of two neighboring states (Chis, Oax; Mickel and Smith, 2004), its presence in similar habitats of central Veracruz is possible.

5. Elaphoglossum mesoamericanum A. Rojas

Note: This species was recorded for Veracruz in Rojas-Alvarado (2003) and Tejero-Díez *et al.* (2011); the cited vouchers (*G. Castillo C. & F. Vázquez B. 1469*, F; *C. Conzatti 798*, GH; *H. Fink 107*, MO; C. *Purpus 1913*, MO) have not been verified and some other vouchers from Chiapas and Oaxaca (e.g., *D. Breedlove & A. Smith 21840*, NY; *J. Mickel & S. Leonard 5226*, MEXU, NY) are cited in Mickel and Smith (2004) under *E. yourkeorum* Mickel, which thus might be a synonym of *E. mesoamericanum* as indicated by Tejero-Díez *et al.* (2011). As both species are very similar to *E. sartorii* (Liebm.) Mickel, a species already recorded for Veracruz in Mickel and Smith (2004), molecular and cytological work, as well as better comparative morphological studies in this species complex are required.

6. Elaphoglossum tejeroanum A. Rojas

Note: This species was mentioned for Veracruz in Rojas-Alvarado (2003) and Tejero-Díez *et al.* (2011); the cited voucher (*J. Beaman & C. Alvarez 5693*, MEXU) has been identified in 1994 by Mickel as *E. sartorii* (Liebm.) Mickel, a species already recorded for Veracruz in Mickel and Smith (2004). Furthermore, Tejero-Díez *et al.* (2011) indicated that *E. tejeroanum* and *E. xanthopodum* Mickel are conspecific, the latter species being also recorded for Veracruz. All three mentioned species are very similar and a molecular study is desirable to confirm their distinctness.

7. Elaphoglossum variabile A. Rojas

Note: This species was mentioned for Veracruz in Rojas-Alvarado (2003) and Tejero-Díez *et al.* (2011); the cited voucher (*D. Barrington 428*, MEXU) has not been found and one voucher from Oaxaca (*J. Mickel & L. Pardue 6521*, NY) was cited in Mickel and Smith (2004) as *E. leebrowniae*.

8. Pecluma alfredii (Rosenst.) M. G. Price var. *cupreolepis* (A. M. Evans) A. R. Sm.

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011). The cited voucher (*M. E. Medina A. & F. Vázquez B. 606*, MEXU) has not been found. Previously, *P. alfredii* was mentioned for Veracruz by Palacios-Ríos (1992), who cited several specimens, all identified by ARS as *P. sursumcurrens* (Copel.) M. G. Price (e.g., *Copeland 127*, BM, MICH, UC, US; *T. B. Croat 39539*, MO, UC). As *P. alfredii* var. *cupreolepis* in Mexico has been reported from pine-oak forests in four neighboring states (Mickel and Smith, 2004), its presence in similar habitats of central Veracruz is possible.

9. Polypodium rzedowskianum Mickel (= *Pleopeltis rzedowskiana* (Mickel) A. R. Sm. & Tejero; see Smith and Tejero-Díez, 2014).

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011). The cited voucher (*M. Sousa 3447*, MEXU) is very similar to collections (e.g., *T. Krömer et al. 2704*, MEXU, UC) made at the same area in Los Tuxtlas identified by ARS as *P. plebeium* Schltdl. & Cham. (= *Pleopeltis plebeia* (Schltdl. & Cham.) A. R. Sm. & Tejero], whereas *P. rzedowskianum* appears to be largely confined to the Pacific coastal states. This highlights the need of molecular and cytological work, as well as better comparative morphological studies in this species complex.

10. Selaginella reflexa Underw.

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011); the cited voucher (*R. Riba et al. 1222-B*, MEXU, UAMIZ) is *S. schiedeana* A. Braun, a species already known from Veracruz. Furthermore, S. *reflexa* usually occurs in dry deciduous forests between 900–2000 m (Mickel and Smith, 2004), a vegetation type not present in the Los Tuxtlas area, where the Riba collection was made.

11. Selaginella rupincola Underw.

Note: This species was recorded for Veracruz in Tejero-Díez *et al.* (2011); however, no voucher was cited and the species is also not mentioned in Palacios-Ríos (1992). As *S. rupincola* in Mexico is widely distributed and has been reported from dry rocky hillsides between 1450–2800 m in six neighboring states (Mickel and Smith, 2004), its presence in similar habitats of central Veracruz is possible.

12. Selaginella sertata Spring

Note: This species was published as new state record for Veracruz in Palacios-Ríos (1992) and Tejero-Díez *et al.* (2011); a cited voucher (e.g., *J. Dorantes L. 110*, MEXU) has not been verified, and others have been identified as *S. schizobasis* Baker (e.g., *S. Sinaca 902*, MEXU, MO; *S. D. Koch et al. 78215*; CHAPA, MEXU, MO, UC), a similar species of a poorly resolved complex. S*elaginella sertata* appears to be largely confined to the Pacific

coastal states and the western part of Mesoamerica, whereas *S. schizobasis* is much more common in eastern Mexico (Mickel and Smith, 2004).

13. Selaginella wrightii Hieron.

Note: This species was published as new state record for Veracruz in Tejero-Díez *et al.* (2011) but has previously been recorded in Palacios-Ríos (1992); the mentioned voucher (*Mueller s.n.*, NY) has not been verified. As *S. wrightii* in Mexico is widely distributed and has been reported from shaded rocks or limestone cliffs at an elevation between 375–2000 m from three neighboring states (Mickel and Smith, 2004), its presence in similar habitats in central Veracruz is possible.

Discussion

Almost 70% of the confirmed 22 new state records result from our recent collections, whereas the others are based on literature and mainly old herbarium specimens. Five of the new records belong to *Elaphoglossum*, four species are filmy ferns, and three are grammitids. Twelve species have a wide distribution ranging from Mexico through Central America to South America, whereas four species are restricted to the United States, Mexico, and/or Central America, and another four are endemic to Mexico. Two species have a disjunct distribution in Mexico and South America. In Veracruz, nine of these new records were made in the Los Tuxtlas area, whereas eight were found for the first time at the slopes of Cofre de Perote and three at the slopes of Pico de Orizaba in central Veracruz. Most taxa occur in humid montane forests (8 species) between 740–2000 m, pine-oak forests (5) between 2000–2500 m or tropical lowland rain forests (5) between 30–600 m. Of the 13 doubtful or unverified species, seven might possibly be confirmed or found during herbarium revisions or field work in the future in Veracruz, whereas six others seem to be misidentified or present taxonomic difficulties.

The 22 new fern records bring the new total for the state of Veracruz to 564 species. Veracruz harbors about 55% of the Mexican pteridophyte flora, and is thus more species-rich than Canada and the continental United States, which harbor 441 species (Wagner and Smith, 1993). Yet it is still less diverse than the neighboring southern states of Chiapas and Oaxaca (Mickel and Smith, 2004). Chiapas has more species of filmy (46) and grammitid ferns (33) than Oaxaca (38 and 26, respectively) and Veracruz (31 and 15), even considering the new records reported here. This corresponds to the latitudinal diversity gradient of pteridophyte diversity, showing a pattern of an increasing number of species per unit area going from the pole toward the equator (Salazar *et al.*, 2015), whereas Costa Rica (surface of ca. 51100 km^2), a country considerably smaller than Veracruz (surface of ca. 72000 km^2), harbors about 1165 species (Moran, 2008).

Five new records belong to *Elaphoglossum*, increasing the number of species present in Veracruz to 32. Another four species mentioned in Tejero-Díez *et al.* (2011) are considered doubtful. This high generic species richness is surpassed

only by *Asplenium* and *Thelypteris* (44 each), which both include only one new record in addition to Mickel and Smith (2004), just like *Polypodium* (30) and *Selaginella* (28), the latter being another genus with four doubtful species. Several of the species that we did not confirm as new records present taxonomic difficulties and thus could not be reliably identified based on the available information. These uncertain species have similar taxa, especially the genera *Elaphoglossum*, *Polypodium* (*Pleopeltis*), and *Selaginella*, which form poorly known species complexes that require molecular and cytological work, as well as better comparative morphological studies.

More than two-thirds of the new records are recent collections made principally in the Los Tuxtlas area and at the slopes of Cofre de Perote, where research projects on fern and epiphyte diversity revealed a high species-richness for both regions. In the Los Tuxtlas Biosphere Reserve, a total of 246 fern species were recorded in a surface of about 1550 km^2 (Acebey *et al.*, 2015), representing 43% of the pteridophytes of Veracruz. Along an elevational transect between 40 and 3520 m at Cofre de Perote, located in central Veracruz, Carvajal-Hernández and Krömer (in press) found 155 fern species in 135 plots of 20 x 20 m (total surface of 0.05 km^2), representing 26% of the state's pteridophyte flora.

The remarkable fern diversity found in relatively small areas in Los Tuxtlas and central Veracruz highlights the importance of both regions for fern conservation. However, the humid montane and pine-oak forests of the central region are still under high anthropogenic pressure and not included inside Veracruz' protected areas network (CONABIO, 2010; Rodríguez-Luna *et al.*, 2011). They should be considered as endangered ecosystems. Consequently, about three-fourths of the grammitid ferns and all of the *Phlegmariurus* species from Veracruz belong to a threatened category, considering the IUCN (2003) regional criteria, mainly because of major habitat destruction within the last decades and a very limited number of known populations either in Los Tuxtlas or central Veracruz (Armenta-Montero *et al.*, 2015; Krömer *et al.*, 2013b).

Finally, our study confirms that continued field work may reveal species new to science or range extensions (e.g., of species formerly considered as endemic to Chiapas or Oaxaca), even in floristically well-known regions. This highlights the need of more floristic inventories in remote and botanically largely unexplored areas, such as Huayacocotla in the north and Uxpanapa in the south of Veracruz. Revisions of national and local herbaria may confirm the identification of several so far unverified species and also discover other specimens that might turn out as new to Veracruz and thereby increase further its outstanding fern diversity.

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Fern-dominated Rock Plant Communities of Tajikistan (Middle Asia)

Arkadiusz Nowak

Laboratory of Geobotany and Plant Conservation, Department of Biosystematics, Opole University, Oleska 48, PL-45-022 Opole; anowak@uni.opole.pl
Department of Biology & Ecology, University of Ostrava, 710 00 Ostrava, Czech Republic

Sylwia Nowak

Laboratory of Geobotany and Plant Conservation, Department of Biosystematics, Opole University, Oleska 48, PL-45-022 Opole

Marcin Nobis

Department of Plant Taxonomy and Phytogeography, Institute of Botany, Jagiellonian University, Kopernika 27, PL-31-501 Kraków; m.nobis@uj.edu.pl
Laboratory of Biodiversity and Ecology, Institute of Biology, Tomsk State University, 36 Lenin Prospekt, Tomsk, 634050, Russia

Agnieszka Nobis

Department of Plant Taxonomy and Phytogeography, Institute of Botany, Jagiellonian University, Kopernika 27, PL-31-501 Kraków

Abstract.—The results of phytosociological research conducted on the fern vegetation of rock crevices and clefts of the Pamir-Alai Mountains in Tajikistan are presented. During field surveys done in 2010–2013, 78 phytosociological relevés were sampled. Plant species were recorded according to the Braun-Blanquet cover-abundance scale. A synopsis of the fern communities of the montane and high altitude zones in Tajikistan is proposed. In the examined vegetation plots, species of 9 ferns, 58 angiosperms and 13 mosses were recorded. The most frequent ferns were: *Cystopteris fragilis, Cheilanthes persica, Asplenium ruta-muraria, Asplenium ceterach, Adiantum capillus-veneris* and *Cryptogramma stelleri.* Six plant associations could be distinguished: *Cheilanthetum persicae, Cryptogrammetum stelleri, Soncho transcaspici-Adiantetum capilli-veneris, Asplenio-Ceterachetum officinarum, Asplenio-Cystopteridetum fragilis,* and *Asplenietum trichomano-rutae-murariae.* The first three associations are described for the first time. The main factors determining the species composition of classified associations are elevation range, microhabitat humidity and moss cover. A minor role is played by the geographic distribution range and rock type.

Key Words.—Pamir-Alai Mountains, chorology, pteridophytes, alpine vegetation, petrophytes

Rock vegetation is unique and very interesting despite not being rich in species, because harsh and extreme chasmophytic habitats can serve as suitable biotopes for many endemic and specialised plant species (Favarger, 1972; Kazakis *et al.*, 2006). In our paper we focus on phytocoenoses dominated by plants able to colonise cracks and fissures of rock faces, stone walls, and bare solid rocky substrates. This group of species is generally defined as chasmophytic, petrophytic or rupicolus plants. Among vascular plants of Tajikistan approximately 800 are considered to be typical petrophytes (Ovchinnikov 1957, 1963). The extreme uniqueness of petrophytic flora is reflected in many phytosociological surveys focused on rupicolous vegetation,

which have been conducted recently in mountainous areas of Europe (e.g., Chytrý, 2009), especially in the Mediterranean region (Bergmeier *et al.*, 2011; Deil *et al.*, 2008; Terzi and D'Amico, 2008). Also, within the area of Tajikistan, floristic analyses reveal the considerable uniqueness of the vascular plant species related to rupicolous habitats (Nowak *et al.*, 2011). However, there are also more widely distributed plant associations of rock crevices and clefts built by ferns and mosses, which generally occur in more shaded and humid sites. Several plant phytocoenoses dominated by fern species, especially from the Mediterranean area, have been described in recent years. Examples are the *Trachelio caeruleae-Adiantetum capilli-veneris* O. Bolós 1957; *Asplenio billotii-Cheilanthetum tinaei* Rivas-Martinez et Costa 1973 corr. Saenz et Rivas-Martinez 1979; *Asplenio ceterach-Cheilanthetum acrosticae* M.T. Santos 1987 in the Iberian Peninsula (Carmona *et al.*, 1997; Costa *et al.*, 2012; Deil *et al.*, 2008;) or *Saxifrago cartilagineae-Asplenietum rutae-murariae* E. Yu. Eromoleva 2007 in the Caucasus (Ermoleva, 2007).

Phytosociological research on Tajikistan's vegetation is still in the initial stages. Just recently, several papers concerning Tajik vegetation classification have been published (e.g. Nowak and Nobis, 2012, 2013; Nowak *et al.*, 2013; Nowak *et al.*, 2014a). The mountains of Tajikistan provide diversified habitat conditions for many petrophytic taxa that are geographically restricted to this country or Middle Asia as a whole, such as *Achoriphragma pinnatifidum* (Kar. & Kir.) Sojak, *Dionisia involucrata* Zapr., *Nanorrhinum ramosissimum* (Wall.) Betsche, *Scutellaria hissarica* B. Fedtsch., *Sergia regelii* (Trautv.) Fed. *Stipa zeravshanica* M. Nobis. No endemic fern species were reported from Tajikistan. All of the rupicolous phytocoenoses have been classified recently into the order *Campanuletalia incanescentis* (Nobis *et al.*, 2013) within the class *Asplenietea rupestris*. The order has been divided into two main alliances: *Asperulo-Poion relaxae* Nobis *et al.* 2013 for alpine rock associations of crevices, walls and ledges and *Caricion koshevnikovii* (Nowak *et al.* 2014) for phytocoenoses of colline and submontane zones. Fern species were noted sporadically with very scarce abundances within these associations. Only *Asplenium ruta-muraria*, *A. trichomanes* and *Cystopteris fragilis* contribute to vegetation plots (Nowak *et al.* 2014 a,b,c),

Although the rich flora of Tajikistan contains over 4550 species (Nobis, 2013; Nobis *et al.*, 2013; Nobis *et al.*, 2014 a,b; Nowak *et al.*, 2011, 2014c; Rasulova, 1991), the pteridophytes comprise only 23 species (Ovczinnikov, 1957; Ryabkova, 1963). Their distribution and ecology is still understudied. Scarce or sometimes only very general information regarding chorology of some pteridophytes is included in the Flora of Tajikistan (Ovczinnikov, 1957) or other regional floras (*e.g.* Kamelin, 1971; Ikonnikov, 1979; Zakirov, 1961). Recently supplementary data on distribution of some pteridophytes in Tajikistan have been published (Nobis *et al.* 2014a). Nevertheless, there are still several species that are known to date from single localities throughout the country (Ovczinnikov 1957), most of these species are included in the red data book of Tajikistan (Kamelin and Ryabkova, 1988).

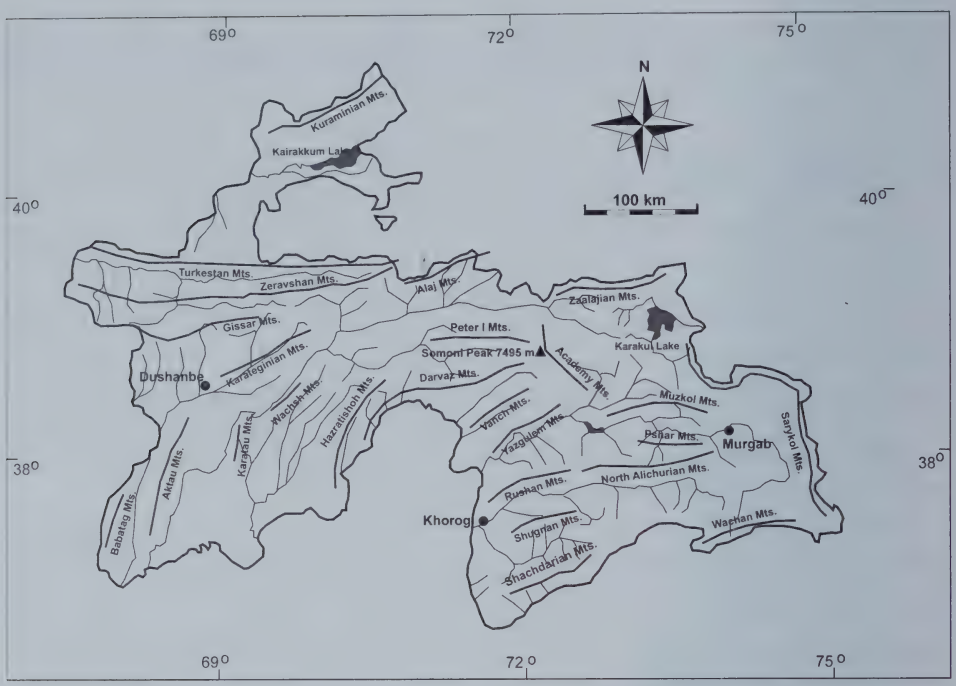

FIG. 1. Map of study area of Tajikistan with main cities, mountain ridges, rivers and lakes.

In this paper, the communities of rock vegetation composed mainly of fern and moss taxa inhabiting all zones of the Pamir Alai Mountains are proposed for the first time. In most cases these phytocoenoses occupy rock crevices and clefts as well as bare rock faces in the Zeravshan, Hissar, Turkestan, Karateginian, Darvaz and Rushan ranges (Fig. 1). These mountains differ considerably in habitat conditions, mainly precipitation and elevation, and provide diversified niches for vegetation. The present contribution is a continuation of the phytosociological research on rock vegetation in Tajikistan (Nobis *et al.*, 2013; Nowak *et al.*, 2014a, b, c). The final aim of the project is to establish a syntaxonomical classification of all phytocoenoses of rock habitats within Tajikistan, especially of areas that are still not fully explored in the extremely inaccessible area of the Pamirian plateau in eastern Tajikistan.

MATERIALS AND METHODS

Study area.—The area of Tajikistan is ca. 143,000 km^2 and extends between latitude 67°31' – 75°14'N and longitude 36°40' – 41°05'E in Middle Asia (Fig. 1). The landscape of high alpine-type mountains dominates the country. More than half of the area is elevated above 3,000 m. According to the bioclimatic classification of the World (Rivas-Martínez *et al.*, 2011), which considers mainly precipitation and temperature values, the study area has to

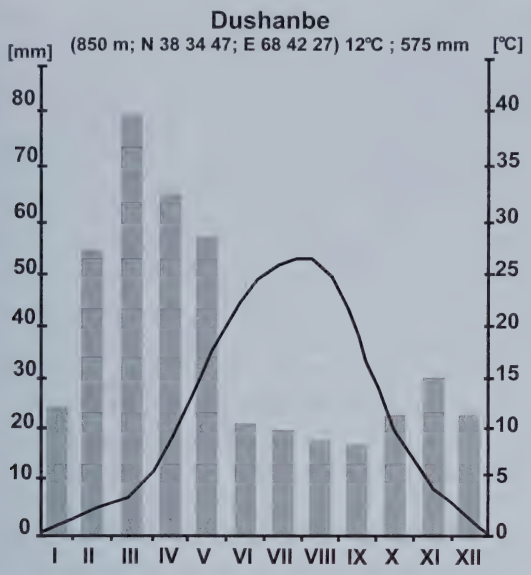

FIG. 2. Climatic diagram of Dushanbe weather station.

be classified within the Mediterranean type of macrobioclimate, characterized by a summer drought lasting for at least two consecutive months in which $P < 2T$ (T - mean monthly temperature). The whole area of Tajikistan with exception of the eastern Pamirian Plateau matches this condition (Fig. 2). The yearly average temperature (12°C) and Compensated Thermicity Index of the study area also classify it to the Mediterranean macrobioclimate. As it is typical for the Mediterranean climate, the area has generally high solar radiation, as well as a low percentage of cloud cover, high-amplitude annual temperatures, low humidity and precipitation, with the exception of the spring period, when there is a considerable amount of rainfall (Fig. 2). In southwestern regions of Tajikistan, the average June temperatures rise to around 30°C. In the temperate zone and alpine elevations the average temperatures in mid summer are between 9.7°C and 13.5°C. Annual precipitation ranges in Tajikistan from ca. 70 mm in mountainous deserts of eastern Pamir and southwestern lowlands of the country to ca. 600 mm in southern slopes of the Hissar Range. The limit of perpetual snow is at an altitude of 3,500–3,600 m in the western Pamir Alai Mts, rising up to about 5,800 m a.s.l. in the highest elevations of eastern Pamir (Latipova, 1968; Narzikulov and Stanjukovich, 1968).

The study was conducted nearly in the whole area of Tajikistan with exception of eastern Pamir. Several mountain ranges were investigated: Hissar Mountains (=Mts), Zeravshan Mts, Turkestan Mts, Hazratishoh Mts, Darvaz Mts, Vanch Mts, Rushan Mts, Peter I Mts, Yazgulem Mts and Karateginian Mts. All of them belong to Pamir Alai mountain system. The studied vegetation plots were located between 670 and 3,150 m a.s.l. on different types of rock substrate, with a pH (measured in 1 n KCl) between 6.5 and 8.5.

Only few studies concerning geological structure have been issued for Tajikistan (*e.g.* Nedzvedskiy, 1968). The middle and higher parts of the Hissar Mts are largely composed of extrusive rocks, mainly granite, granitoid and syenite. Some igneous outcrops also occur in the Darvaz Mts, Kuraminian Mts and in the western Pamir ranges. In the Zeravshan and Turkestan Mts, the Cambrian and Sylurian sediments predominate. The rocks there are generally limestone (micrite limestone, bitumic limestone, marly limestone and dolomitic coral limestone), marble, dolomite, dolomitic shale, clay shale, phyllitic schist and argillaceous slate. Also several metamorphic rocks are present within the study area. The most common are migmatic gneiss, conglomerates and metamorphic mudstones.

Field work and data analyses.—The field research was conducted in 2010–2013. During the surveys 78 phytosociological relevés were taken. The plot size was delimited in such a way as to represent full floristic composition of the phytocoenosis and varied from 0.3 to 2 m^2 depending on plant density and homogeneity of vegetation cover. For each vegetation plot all vascular plants and cryptogams were recorded with the use of Braun-Blanquet approach (Braun-Blanquet, 1964). The 7-degree cover-abundance scale was used. Geographical coordinates, elevation above sea level, aspect and slope inclination were noted for each relevé. The rock type was determined by analysing the lithology, pore geometry, mineralogical components, texture, permeability, hardness and pH by a professional geologist (see acknowledgments). Hydrogen ion concentrations were measured in aqueous rock solution using the ELMETRON CP-105 pH meter.

All the relevés were stored in a database using the JUICE program (Tichý, 2002). A TWINSPAN analysis (Hill, 1979) was used to perform the preliminary classification of communities. As relevés data showed a clear unimodal response, allowing us to use a Detrended Correspondence Analysis (DCA) with the floristic data set (presence-absence data, no downweighting of rare species) to check the floristic-sociological classification and to show the relationships between the groups. For the ordination, CANOCO for Windows 4.5 was used (Ter Braak and Šmilauer, 2002).

Vegetation classification follows the sorted table approach of Braun-Blanquet (1964). Species constancies are given in classes I–V (Dierschke, 1994). For species present in less than 8 relevés, the absolute number of species occurrences was specified. Newly presented syntaxa, described as order, alliance or associations were proposed according to the International Code of Phytosociological Nomenclature (Weber *et al.*, 2000). While distinguishing and ranking the association, Dimopoulos *et al.* (1997), Ermolaeva (2007), Hein *et al.* (1998), Nowak *et al.* (2014a,b) and Valachovič *et al.* (1997), were taken into account. The association concept follows Willner (2006). The presented communities are arranged into a syntaxonomic overview. Species nomenclature followed mainly Czerepanov (1995). Plant material collected during field studies was deposited in the Herbarium of Middle Asia Mountains, hosted in OPUN (Opole University, Poland) and KRA (Jagiellonian University, Poland).

Results

The number of vascular plants recorded in the relevés totaled 67. The most frequent species were six ferns and nine mosses (Table 1). As it was revealed in samples of vegetation plots, mosses were more frequent, but not very abundant in the sampled phytocoenoses (Fig. 3). Most species are cosmopolitan or widely distributed taxa in the northern hemisphere. Only few angiosperm species (e.g. *Anemone zeravshanica* or *Aquilegia vicaria*) but none of the fern species are Tajikistan endemics. Not all of the species noted in relevés are typical for rock crevice vegetation. In the studied plots some ruderal species or plants typical for nitrophilous and often anthropogenic habitats were present: *Poa bulbosa*, *Bromus tectorum*, *Phleum graecum*, *Galium aparine* or *Parietaria serbica*. Species typical for alpine rock faces have also been recorded (*Campanula incanescens, C. lehmanniana*, *Pentanema albertoregelia*, *Paraquilegia caespitosa*) as well as plants typical of submontane chasmophytic vegetation (e.g. *Carex koshevnikovii*, *Scutellaria zapriagaevii*, *Spiraea baldshuanica*), mountain tall-herbs (e.g. *Cicerbita zeravshanica*) or deciduous forests (e.g. *Impatiens parviflora*).

As a result of the numerical classification of all relevés of rock crevices, six associations of fern dominated rock plant communities were distinguished. Three of them were defined in the present contribution by original diagnoses based on species composition: *Cheilanthetum persicae*, *Cryptogrammetum stelleri*, and *Soncho transcaspici-Adiantetum capilli-veneris* (Fig. 4). Due to considerable differences in species composition, the associations have to be classified into different higher syntaxa. Most of them were assessed as members of three different alliances within the *Asplenietea trichomanis* class. One association, the *Soncho transcaspici-Adiantetum capilli-veneris* was assigned to *Adiantetea capilli-veneris* due to considerably different habitat characteristic and species composition (especially mosses).

Based on this study, we propose the classification of fern dominated rock plant communities of the Pamir Alai Mts in Tajikistan (Tables 2–6) with the following six associations.

1. *Asplenio-Cystopteridetum fragilis* Oberdorfer 1938

Diagnostic species: *Cystopteris fragilis, Asplenium viride*

The plant association built by *Cystopteris fragilis* was found in many locations in the Hissar, Zeravshan, Turkestan, Karateginian, Rushan, Darvaz, Shugnan and Vanch Mts. This phytocoenosis has been found on different rock substrates, mainly on limestone, marble and schist, but also on dolomite and conglomerate rocks (pH 6.8–7.7). The association occurs most frequently in crevices of different size and various soil amounts. The phytocoenosis develops on steep rock walls with mean inclination of approximately 85°, within a wide elevational range in colline, montane and alpine zones at elevations of approx. 1,300–3,200 m a.s.l. (mean ca. 2,000). Occasionally the community was found on rock taluses sloping gently down. *Asplenio-Cystopteridetum fragilis* is

TABLE 1. Frequencies of the most common vascular plants and mosses of fern-dominated rock plant communities of Tajikistan.

Vascular plant species	Number of occurrences	Moss species	Number of occurrences
Cystopteris fragilis	23	*Tortula muralis*	33
Cheilanthes persica	19	*Encalypta vulgaris*	27
Asplenium ruta-muraria	16	*Grimmia pulvinata*	15
Ceterach officinarum	13	*Hygrohypnum luridum*	14
Adiantum capillus-veneris	9	*Brachythecium mildeanum*	12
Cryptogramma stelleri	8	*Bryum pseudotriquetrum*	12
Campanula incanescens	7	*Brachythecium albicans*	9
Cortusa turkestanica	7	*Tortula inermis*	9
Poa bulbosa	6	*Amblystegium varium*	8

related to northwestern and northern expositions with moderate insolation. The mean value of herb layer cover is about 40% ranging from 20 to 65% (Fig. 3, Table 1). Mosses enter the plots relatively rarely and have the mean cover value of approximately 10%. The most abundant and constant vascular plant species within the plots of *Asplenio-Cystopteridetum fragilis* are *Poa relaxa* and *Campanula incanescens*. Most abundant and frequent mosses are *Tortula muralis*, *Grimmia pulvinata* and *Syntrichia ruralis*. Within the association two variants could be distinguished depending on the occurrence of *Asplenium viride* and *Gymnocarpium fedtschenkoanum*. *Asplenium viride* was found in few plots in the Maykhura valley in the Hissar Mts as a codominant species on solid marl face with northern exposure *Gymnocarpium fedtschenkoanum*, similarily to the European populations, inhabits in Tajikistan mainly limestone rock faces. The species contribute to *Asplenio-Cystopteridetum fragilis* in few places in the Zeravshan Mts.

2. *Asplenietum trichomano-rutae-murariae* Tüxen 1937

Diagnostic species: *Asplenium ruta-muraria, Asplenium trichomanes*

In Pamir Alai Mts the association is built mainly by *Asplenium ruta-muraria*. This phytocoenosis has been found in most cases on limestone and

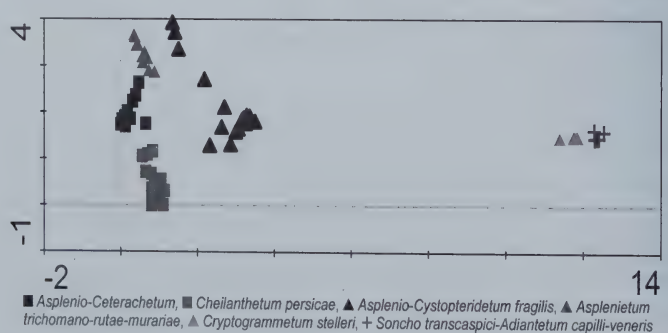

FIG. 3. DCA ordination of relevés of rock plant communities (N=182).

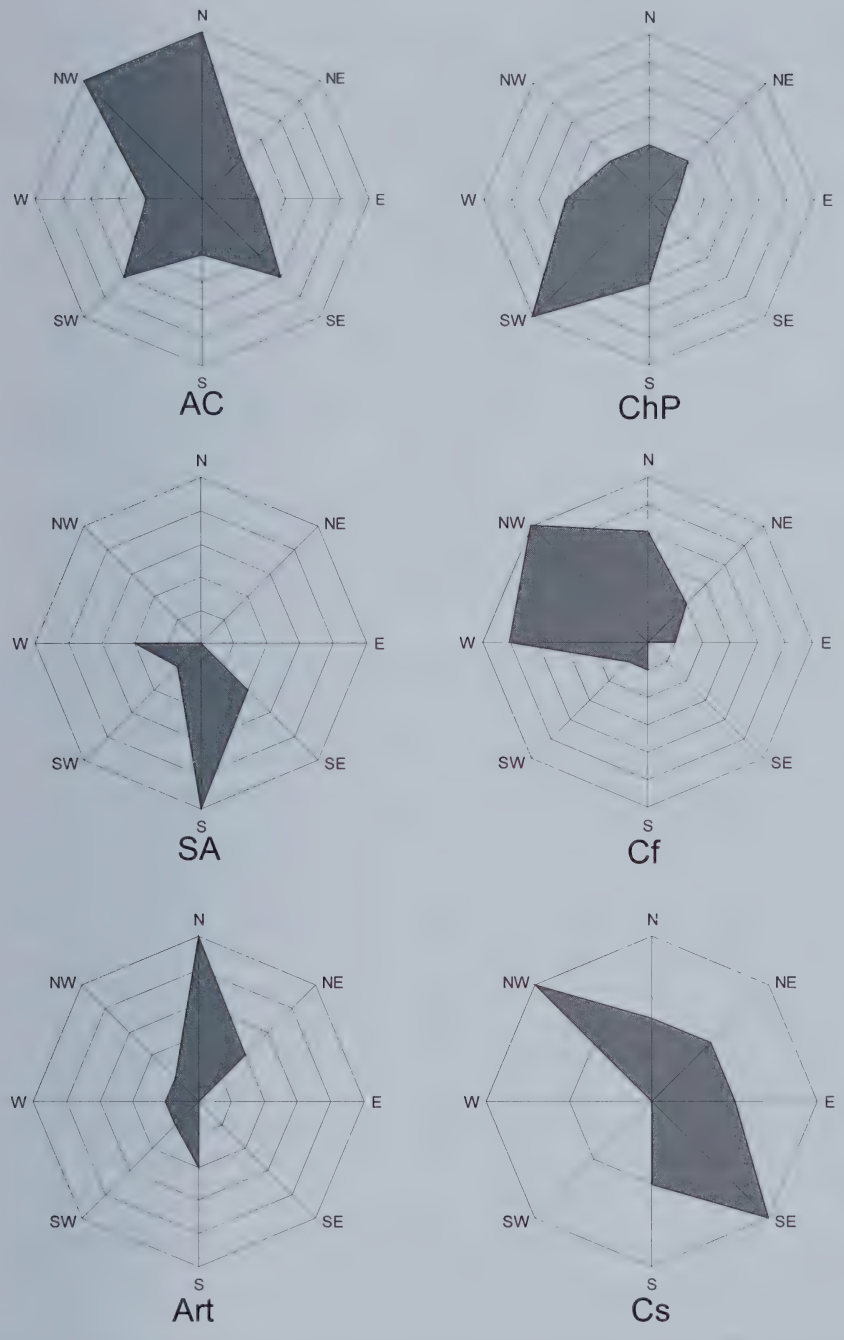

FIG. 4. Frequencies of rock expositions of six fern-dominated rock plant associations.

TABLE 2. Synopsis of fern-dominated vegetation of rock crevices of the Pamir Alai Mts in Tajikistan.

Rank	Syntaxon	Abbreviation
Class	*Asplenietea trichomanis* (Br.-Bl. in Meier et Br.-Bl. 1939) Oberdorfer 1977	
Order	*Potentilletalia caulescentis* Br.-Bl. in Br.-Bl. et Jenny 1926	
Alliance	*Cystopteridion fragilis* Richard 1972	
Association	**1.** *Asplenio-Cystopteridetum fragilis* Oberdorfer 1938 - variant with *Asplenium viride* - variant with *Gymnocarpium fedtschenkoanum*	Cf
Association	**2.** *Asplenietum trichomano-rutae-murariae* Tüxen 1937	Art
Order	*Tortulo-Cymbalarietalia* Segal 1969	
Alliance	*Cymbalario-Asplenion* Segal 1969 em. Mucina 1993	
Association	**3.** *Asplenio fontani-Ceterachetum officinarum* Gillet ex Ferrez 2009	AC
Association	**4.** *Cheilanthetum persicae* ass. nova	ChP
Class	*Adiantetea capilli-veneris* Br.-Bl. in Br.-Bl., Roussine & Nègre 1952	
Order	*Adiantetalia capilli-veneris* Br.-Bl. ex Horvatiç 1934	
Alliance	*Adiantion capillus-veneris* Br.-Bl. ex Horvatiç 1934	
Association	**5.** *Soncho transcaspici-Adiantetum capilli-veneris* ass. nova	SA
Association	**6.** *Cryptogrammetum stelleri* ass. nova	Cs

dolomite, rarely on schist and conglomerate rocks (pH 6.8–7.8), mainly in the Zeravshan, Hissar, Darvaz and Rushan ranges (Fig. 1). In the Pastruddaria River valley the community was also recorded on the northern side of solitary boulders. The association occurs mostly on rather solid rocks, with various inclinations, ranging from 50° to overhanging rock ledges with 140° value (mean ca. 85°, Fig. 3) and occasionally occurs on friable substrates. *Asplenietum trichomano-rutae-murariae* develops on relatively high elevations in the alpine zone with cold microclimate; elevational range 1,450 to 2,600 m a.s.l. (mean approximately 1,850). The mean value of herb layer cover was relatively low and did not exceed 20% (ranging from 10–40%; Fig. 3). The mean value of moss cover in surveyed plots was approximately 5% on average, however some plots were characterized by an extremely abundant moss layer, up to 65%. The most abundant and constant species of mosses are *Tortula muralis* and *Encalypta vulgaris*. Vegetation plots have few vascular species and are dominated by the main diagnostic species that are scarce. In some plots *Cystopteris fragilis*, *Callipeltis cucullaris*, *Campanula incanescens* and *Carex koshevnikovii* were observed.

3. *Asplenio fontani-Ceterachetum officinarum* Gillet ex Ferrez 2009

Diagnostic species: *Asplenium ceterach* (=*Ceterach officinarum*)

In Tajikistan *Asplenium ceterach* was reported from several locations in western part of the country. The distribution of the association is restricted to lower and middle elevations of the Zeravshan, Hissar and Karateginian ranges. The species occur generally on undisturbed sites. Although *Asplenium pseudofontanum* (recorded in Pamir Alai) is treated by some of researchers

TABLE 3. Associations of *Asplenio-Cystopteridetum fragilis* and *Asplenietum trichomano-rutae-murariae* in the Pamir Alai Mountains in Tajikistan.

Relevé number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Constancy	Constancy
day	15	15	15	12	12	3	9	13	12	4	12	12	18	14	9	18	18	18	9	9	13	12	17	19	19	18	18	9	9	8		
Date: month	6	6	6	6	5	6	6	6	6	6	6	6	6	6	6	8	8	8	6	6	6	6	8	8	8	8	8	6	6	6		
year	10	10	10	12	11	11	12	12	11	11	11	12	12	11	12	13	13	13	12	12	11	12	12	12	12	13	13	12	12	13		
Aspect	NW	NW	E	N	N	W	W	W	NW	NW	N	NE	S	NE	W	NW	N	NW	W	S	N	NW	N	N	N	NE	NE	SW	S	N		
Inclination (degrees)	85	80	75	90	90	90	85	90	75	85	90	80	90	30	120	80	85	85	100	140	65	75	85	55	55	120	130	85	75	130		
Altitude (m)	1620	1620	1632	2425	1850	2465	1980	1465	1710	2173	1710	2410	3155	2384	1980	2100	2095	2090	1950	1820	1864	2425	2550	1865	1865	1930	1931	1790	1825	1856		
Cover of herb layer (%)	75	60	80	55	55	25	55	30	40	35	30	35	15	20	60	30	15	25	15	30	15	15	15	30	20	20	40	20	20	35		
Cover of moss layer (%)	0.5	5	5	10	10	30	10	5	15	5	5	15	40	10	5	10	-	-	10	0.5	5	5	20	20	10	60	55	5	0.5	0.5		
Relevé area (m2)	1	1	0.5	0.5	0.5	1	0.5	0.5	1	1	0.5	0.5	0.5	4	1	0.5	0.5	0.5	0.5	1	1	0.5	0.5	1	1	0.5	0.5	1	1	0.5		
pH	6.8	6.8	6.8	7.7	7.7	7.5	7.6	7.3	7.3	7.5	7.3	7.7	7.5	7.5	7.6	7.6	7.6	7.6	7.2	7.7	7.5	7.5	7.8	7.6	7.6	7.8	7.8	7.4	7.5	6.8		
Locality	DU	DU	DU	7L	7L	IK	MG	MS	MS	IK	MS	7L	DW	SG	MG	MA	MA	MA	MG	AN	AN	7L	PO	CH	MG	MA	MA	TK	AN	TU		
Number of species	4	3	4	4	4	2	5	5	7	3	3	3	4	7	7	6	3	3	4	3	4	3	4	9	5	4	3	5	5	3	1–18	19–30
Diagnostic species																																
Ass. *Asplenio -Cystopteridetum fragilis*																																
Cystopteris fragilis	4	4	4	4	4	3	3	3	3	3	3	3	2	2	1	2	+	+	.	.	.	.	+	+	.	.	.	.	+	.	V	II
Gymnocarpium fedtschenkoanum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	2	.	.	+	.	.	.	.	.	.	.	.	.	.	.	I	I
Asplenium viridae	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	2	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Ass. *Asplenietum trichomano-rutae-murariae*																																
Asplenium ruta-muraria	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	1	1	2	3	2	2	2	2	2	2	3	2	2	3	I	V
All. *Cystopteridion fragilis* et O. *Potentilletalia caulescentis* et Cl. *Asplenietea trichomanis*																																
Tortula muralis d	.	1	1	+	+	.	1	1	2	1	1	+	1	.	1	.	.	.	.	.	.	+	1	1	1	4	4	1	+	.	IV	IV
Grimmia pulvinata d	.	.	.	1	1	3	2	.	.	.	.	.	.	1	+	1	.	.	.	.	1	.	.	.	.	.	+	.	.	.	II	I
Syntrichia ruralis d	.	.	+	1	.	.	.	.	.	.	.	2	3	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	II	-
Campanula incanescens	1	.	.	.	.	.	1	+	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	II	I
Poa relaxa	.	.	.	.	.	.	.	.	1	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Brachythecium albicans d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	+	.	.	.	.	-	I
Penthathema albertoregelia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	-	I
Sporadic species: *Achoriphragma pinnatifidum* 15; *Anemone zeravshanica* 8; *Asplenium trichomanes* 30(1); *Campanula lehmanniana* 16; *Carex koshevnikovii* 28; *Paraquilegia caespitosa* 7; *Parietaria judaica* 3(1); *Tortula inermis* d 5(1).																																
Others																																
Encalypta vulgaris d	+	.	.	.	.	.	.	.	.	.	.	.	1	1	+	.	.	.	1	+	+	1	2	2	1	1	.	.	.	+	II	IV
Gagea sp.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	I	I
Poa bulbosa	.	.	.	.	.	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Parietaria serbica	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	+	.	.	.	.	-	I
Callipeltis cucullaris	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	.	.	-	I
Sporadic species: *Aulacospermum dichotomum* 24; *Dasiphora parviflora* 24(1); *Drepanocaryum* sp. 9(1); *Festuca valesiaca* 10(1); *Impatiens parviflora* 1; *Lepyrodiclis stellarioides* 9; *Paraquilegia uniflora* 2; *Rhodiola pamiroalaica* 14; *Silene brahuica* 14(1); *Taraxacum* sp. 29; *Thalictrum alpinum* 9; *Veronica capillipes* 24; *Veronica rubrifolia* 24.																																

Explanations: 7L - 7th lake in Seven Lake Valley, AN - Anzob, CH - Chore, DU - near Dukdon pass, DW - Darwaz pass, IK - Iskander-Kul Lake, MA - Maykhura valley, MG - Margeb, MS - Mazari-Sharif, PO - Poymazor, SG - Sarytag, TK - Takfon, TU - Tushark.

TABLE 4. Associations of *Cheilanthetum persicae* and *Asplenio-Ceterachetum officinarum* in the Pamir Alai Mountains in Tajikistan.

Relevé number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Constancy	Constancy
day	13	13	21	11	16	18	12	2	17	17	26	17	7	11	18	17	18	9	11	14	8	12	12	12	16	11	11	11	12	17	18		
Date: month	6	6	6	5	6	8	5	6	6	6	8	6	9	5	8	6	8	6	5	6	6	6	6	6	6	5	5	5	5	8	8		
year	10	10	10	11	12	13	11	11	11	11	13	11	11	11	13	11	13	12	11	12	12	10	11	11	11	11	11	11	11	13	13		
Aspect	W	W	N	NE	S	W	N	SE	SW	SW	S	SW	SW	SW	NW	SW	S	SW	NE	NW	SW	W	N	N	N	NE	SE	SE	NW	SW	NW		
Inclination (degrees)	90	90	90	90	90	130	65	80	85	110	95	110	85	90	130	85	85	85	85	45	110	75	90	90	75	85	85	85	65	120	80		
Altitude (m)	1720	1720	1420	761	1180	1430	1073	2150	969	975	1144	975	2805	1037	1427	969	1361	1790	956	1620	1044	1655	1710	1710	1323	956	956	956	1073	1403	1875		
Cover of shrub layer (%)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-		
Cover of herb layer (%)	20	35	30	20	15	55	30	30	15	35	30	55	70	10	30	25	25	20	70	80	75	20	15	30	30	20	5	15	15	30	35		
Cover of moss layer (%)	15	20	10	5	10	5	15	10	10	5	20	20	25	10	10	5	5	5	5	5	15	5	5	30	35	45	35	20	25	15	25		
Relevé area (m2)	1	1	0.5	0.5	0.5	0.5	1	2	1	1	0.5	1	2	0.5	0.5	1	0.5	1	1	0.5	0.5	1	1	0.5	1	1	0.5	0.5	1	0.5	0.5		
pH	7.8	8.4	7.7	7.8	8.2	8	8.2	7.8	8.4	8.4	8.4	8.5	8	8.2	8.2	8.5	7.8	8.4	8.2	7.4	7.2	7.2	8	7.8	8.2	7.8	8	7.8	8.2	8.4	7.5		
Locality	SH	SH	VE	OK	VR	HR	KH	IK	HR	HO	ZA	GU	ST	HO	HR	HO	HR	TK	HO	ZT	VR	MO	MS	MS	PU	HO	HO	HO	VR	PU	TU		
Number of species	6	8	4	7	5	5	4	4	3	5	8	4	3	4	4	5	6	4	3	4	5	4	2	6	4	2	4	3	2	5	4	1–19	20–31
Diagnostic species																																	
Ass. *Cheilanthetum persicae*																																	
Cheilanthes persica	2	3	3	2	2	4	3	3	2	3	3	4	4	1	3	2	2	2	4	.	.	.	.	.	.	.	.	.	.	.	.	V	-
Ass. *Asplenio-Ceterachetum*																																	
Asplenium ceterach	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	5	4	2	2	3	3	2	1	2	2	3	3	I	V
All. *Cymbalario-Asplenion* et O. *Tortulo-Cymbalarietalia* et Cl. *Asplenietea trichomanis*																																	
Tortula muralis d	.	.	.	.	.	.	.	.	.	.	1	2	2	1	1	.	.	.	.	.	.	.	.	1	2	3	3	2	3	2	3	II	IV
Tortula inermis d	2	2	.	.	.	.	.	.	.	1	+	.	.	.	.	1	1	1	1	.	.	.	.	.	.	.	.	.	.	.	.	III	-
Brachythecium albicans d	.	.	1	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	+	.	1	2	.	.	.	.	.	.	.	.	I	II
Grimmia pulvinata d	.	.	.	.	.	.	.	.	1	+	2	1	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	II	I
Hypnum cupressiforme d	.	+	.	.	.	.	.	.	.	.	.	.	.	1	1	+	.	.	.	.	.	.	.	.	.	.	.	+	.	.	+	.	II	I
Carex koshevnikovii	.	.	.	.	.	.	.	.	.	.	+	+	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	II	-
Penthathema albertoregelia	+	1	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Syntrichia ruralis d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	I	I
Campanula incanescens	.	+	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Sporadic species: *Asperula laevis* 4; *Campanula lehmanniana* 4; *Parietaria judaica* 8; *Poa relaxa* 31(1); *Scutellaria zapriagajevii* 16; *Spiraea baldshuanica* b 30(1).																																	
Others																																	
Encalypta vulgaris d	.	1	1	+	1	1	2	1	1	.	.	.	1	.	.	.	.	.	.	.	2	1	.	1	.	.	1	.	.	+	.	III	III
Poa bulbosa	+	.	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	I	I
Geranium divaricatum	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	+	.	+	+	.	.	.	.	.	.	.	.	.	.	I	I
Gagea sp.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	I	I
Bryum argenteum d	.	.	.	.	.	.	+	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Bromus tectorum	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	I	I
Origanum tythanthum	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Sanguisorba alpina	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Pseudosedum condensatum	.	.	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	-
Poa trivialis	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	I	I
Sporadic species: *Artemisia absinthium* 2; *Conringia planisiliqua* 18; *Galium aparine* 7; *Galium spurium* 20; *Geranium rotundifolium* 22; *Impatiens parviflora* 19; *Parietaria serbica* 4(1); *Phleum graecum* 15; *Pimpinella peregrina* 21; *Silene ladygini* 4; *Sisymbrium loeselii* 5; *Taraxacum* sp. 1;																																	
Explanations: GU - Guraha, HO - Horbogh, HR - Harangon, IK - Iskander-Kul, KH - Khondara, MO - Mogien, MS - Mazari-Sharif, SH - Shing, ST - Shahristan Pass, TK - Takfon, TU - Tushark, VE - Veshan, OK - Obi-Kiik, PU - Pugus, VR - Varzob, ZA - Zangerya, ZT - Zimtut																																	

TABLE 5. Associations of *Cryptogrammetum stelleri* and *Soncho transcaspici-Adiantetum capilli-veneris* in the Pamir Alai Mountains in Tajikistan.

Relevé number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Constancy	Constancy
day	14	14	14	14	14	14	14	14	14	11	11	1	26	26	18	16	18		
Date: month	8	8	8	8	8	8	8	8	6	6	6	6	8	8	8	6	8		
year	12	12	12	12	12	12	12	12	10	12	12	11	13	13	13	10	13		
Aspect	N	S	NE	SE	SE	E	NW	NW	S	W	W	SE	SE	S	S	S	S		
Inclination (degrees)	80	55	80	70	70	70	70	60	120	85	85	90	100	160	90	150	85		
Altitude (m)	2100	2220	2950	2950	2220	2950	2100	2100	995	1430	1430	673	1284	1284	1393	1050	1393		
Cover of herb layer (%)	20	20	20	60	60	45	50	35	95	70	85	35	75	70	75	60	90		
Cover of moss layer (%)	20	15	25	20	20	20	20	20	5	5	10	20	10	5	20	10	5		
Relevé area (m2)	0.4	0.3	0.4	0.3	0.3	0.4	0.4	0.4	4	1	5	2	10	10	10	3	10		
pH	7.8	7.5	7.5	-	7.5	7.5	7.8	7.8	6.5	7.2	7.2	7.4	7.5	7.5	7.6	7	7.5		
Locality	MA	MG	KA	KA	MG	KA	MA	MA	GU	KU	KU	GL	AD	AD	BA	PN	BA		
Number of species	6	6	6	7	7	6	5	7	5	3	8	4	5	5	7	6	5	1–8	9–17
Diagnostic species																			
Ass. *Cryptogrammetum stelleri*																			
Cryptogramma stelleri	2	2	2	4	4	3	3	3	.	.	.	.	.	.	.	.	.	V	-
Ass. *Soncho transcaspici-Adiantetum capilli-veneris*																			
Adiantum capillus-veneris	.	.	.	.	.	.	.	.	5	4	4	3	4	4	4	4	5	-	V
Sonchus transcaspicus	.	.	.	.	.	.	.	.	+	+	1	+	+	.	.	+	.	-	IV
All. *Adiantion capillis-veneris* et O. *Adiantetalia capilli-veneris* et Cl. *Adiantetea capilli-veneris*																			
Brachythecium mildeanum d	.	1	1	1	+	1	1	1	+	1	1	.	+	+	.	.	.	V	III
Amblystegium varium d	+	.	1	1	.	.	.	.	.	.	+	1	1	1	+	.	.	II	III
Others																			
Hygrohypnum luridum d	1	1	1	+	1	+	.	2	1	.	1	2	1	+	2	1	.	V	IV
Bryum pseudotriquetrum d	1	1	2	2	2	2	2	+	.	.	.	.	.	+	1	1	1	V	III
Cortusa matthioli	+	+	+	+	+	2	2	.	.	.	.	.	.	.	.	.	.	V	-
Aquilegia vicaria	.	.	.	+	+	+	+	+	.	.	.	.	.	.	.	.	.	IV	-
Brachythecium rivulare d	2	1	.	.	+	.	.	.	.	.	.	.	.	.	.	+	.	II	I
Erianthus ravennae	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	+	-	II

Sporadic species: *Calamagrostis dubium* 11(1); *Cicerbita seravshanica* 11; *Corydalis kashgarica* 16; *Cystopteris fragilis* 8; *Epilobium hirsutum* 15(1); *Gentianopsis vvedensky* 17; *Impatiens parviflora* 8; *Inula macrolepis* 17(1); *Juncus articulatus* 15; *Reseda lutea* 9; *Rubus caesius* c 11; *Schoenus nigricans* 13(1).

Explanations: AD - Ab Darrah, BA - Barou, GL - Guldava, GU - Gusar, KA - Kaznok, KU - Kumarg, MA - Maykhura, MG - Margeb, PN - Panjikent

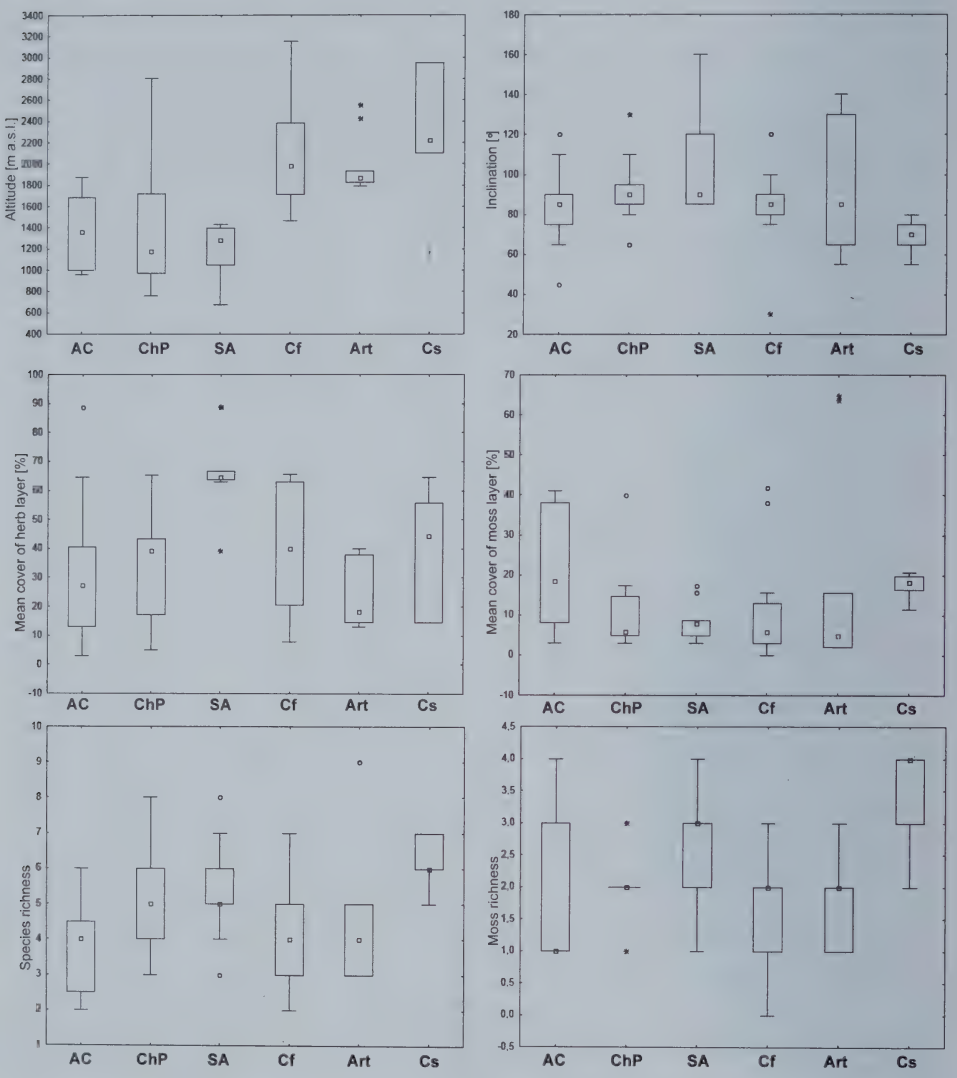

FIG. 5. Elevational distribution, cliff inclinations, cover values of herb and moss layer and species richness of six fern-dominated rock plant associations: *Cheilanthetum persicae*, *Cryptogrammetum stelleri*, *Soncho transcaspici-Adiantetum capilli-veneris*, *Asplenio-Ceterachetum officinarum*, *Asplenio-Cystopteridetum fragilis*, and *Asplenietum trichomano-rutae-murariae*). The top and bottom of each box are the 75% and 25% quartiles. The dots and asterisks indicate maximum and extreme values.

as a subspecies of *A. fontanum*, the nominal taxon does not occur in the country. *Asplenium ceterach* occurs most frequently in alkaline rock substrates mainly on limestone and dolomite (pH 7.2–8.4). This vegetation type was found in rock coarse crevices, often under the overhanging ledges. It develops generally on vertical walls, sometimes hanging over, with a mean inclination value close to 90° ; Fig. 3). This association occurs generally on northwestern and northern

expositions (Fig. 5), however it was also present on southern sides of shaded rock faces. It was recorded at different elevations in submontane and colline zones (Fig. 3), within the elevation range of 900 to 1,900 m a.s.l. (mean ca. 1,300). Within the sampled plots, between two and six taxa of vascular species were recorded (mean ca. 4). The scarcity of species is also related to a low cover of herbs, which is approximately 25%, ranging from 5% to 65% (Fig. 3). The moss layer of the surveyed plots is relatively more abundant with a cover value between 5% and 40% and a mean cover value close to 20%. In addition to diagnostic species, the angiosperms *Geranium divaricatum*, *G. rotundifolium* and *Galium spurium* and the mosses *Brachythecium albicans*, *Encalypta vulgaris*, *Grimmia pulvinata*, *Syntrichia ruralis*, *Hypnum cupressiforme* and *Tortula muralis* have been found. The last species is the most frequent in the patches of the association.

4. *Cheilanthetum persicae* assoc. nova

Typus relevé: Table 4, rel. 1, holotypus hoc loco

Diagnostic species: *Cheilanthes persica*

The phytocoenosis of *Cheilanthetum persicae* has been found in several locations in Hissar, Zeravshan, Turkestan and Karateginian Mts (Fig. 1) at altitudes of 1,000 to 1,700 m a.s.l. (mean approx. 1,200; Fig. 3, Table 2). The association often occurs in limestone and dolomite rocks (pH 7.7–8.5) with coarse crevices and clefts. It was also spotted on man-made walls. The *Cheilanthetum persicae* was found mainly on southwestern exposures with inclinations of mean value approximately 90°. In comparison to other ferns communities, the patches of *Cheilanthethum persicae* occupy less shadowed places, sometimes overgrowing very sunny walls or faces on western or southeastern exposures. The association is characterised by a moderate abundance of vegetation cover. The total cover of the herb layer generally was between 20 and 45% with mean values of around 40% (Table 2, Fig. 3). Mosses quite insignificantly contribute to the association when compared to other communities. Their total cover value varied between 3–18%, occasionally reaching up to 40% (Fig. 3). The phytocoenosis is characterised by a low number of three to eight vascular plant species per relevé (mean ca. 5) and one to three mosses (mean 2). Among the vascular plant species *Carex koshevnikovii*, *Geranium divaricatum* and *Poa bulbosa* had the highest values of constancy and abundance. *Tortula inermis*, *T. muralis*, *Hypnum cupressiforme*, *Brachythecium albicans* and *Encalypta vulgaris* were the most abundant mosses.

5. *Soncho transcaspici-Adiantetum capilli-veneris* assoc. nova

Typus relevé: Table 5, rel. 10, holotypus hoc loco

Diagnostic species: *Adiantum capillus-veneris, Hygrohypnum luridum*

Soncho transcaspici-Adiantetum capilli-veneris was found on five locations in the Zeravshan, Hissar, Turkestan, Darvaz and Shugnan ranges. The

TABLE 6. The synoptic table for fern-dominated chasmophytic associations in Tajikistan. Explanations: 1 – *Asplenio-Cystopteridetum fragilis*; 2 – *Asplenietum trichomano-rutae-murariae*; 3 – *Cheilanthetum persicae*; 4 – *Asplenio-Ceterachetum*; 5 – *Cryptogrammetum stelleri*; 6 - *Soncho transcaspici-Adiantetum capilli-veneris*.

Syntaxonomic unit	1	2	3	4	5	6
Number of relevés	18	12	19	12	8	9
Average species number in relevé	4	4	5	4	6	5
Ass. *Asplenio-Cystopteridetum fragilis*						
Cystopteris fragilis	100	25	.	.	13	.
Gymnocarpium fedtschenkoanum	11	8	.	.	.	.
Asplenium viridae	11	.	.	.	.	.
Ass. *Asplenietum trichomano-rutae-murariae*						
Asplenium ruta-muraria	17	100	.	.	.	.
Ass. *Cheilanthetum persicae*						
Cheilanthes persica	.	.	100	.	.	.
Ass. *Asplenio-Ceterachetum*						
Asplenium ceterach	.	.	5	100	.	.
Ass. *Cryptogrammetum stelleri*						
Cryptogramma stelleri	.	.	.	.	100	.
Ass. *Soncho transcaspici-Adiantetum capilli-veneris*						
Adiantum capillus-veneris	.	.	.	.	.	100
Sonchus transcaspicus	.	.	.	.	.	67
Cl. *Asplenietea trichomanis*						
Tortula muralis d	67	67	26	67	.	.
Grimmia pulvinata d	39	17	21	17	.	.
Syntrichia ruralis d	28	.	5	8	.	.
Campanula incanescens	22	8	11	.	.	.
Brachythecium albicans d	.	17	16	33	.	.
Tortula inermis d	6	.	42	.	.	.
Hypnum cupressiforme d	.	.	21	17	.	.
Penthathema albertoregelia	.	17	16	.	.	.
Carex koshevnikovii	.	8	21	.	.	.
Poa relaxa	17	.	.	8	.	.
Campanula lehmanniana	6	.	5	.	.	.
Parietaria judaica	6	.	5	.	.	.
Achoriphragma pinnatifidum	6	.	.	.	.	.
Anemone zeravshanica	6	.	.	.	.	.
Paraquilegia caespitosa	6	.	.	.	.	.
Asplenium trichomanes	.	8	.	.	.	.
Spiraea baldshuanica b	.	.	.	8	.	.
Asperula laevis	.	.	5	.	.	.
Scutellaria zapriagajevii	.	.	5	.	.	.
Cl. *Adiantetea capilli-veneris*						
Brachythecium mildeanum d	.	.	.	.	88	56
Amblystegium varium d	.	.	.	.	38	56
Others						
Encalypta vulgaris d	22	75	47	42	.	.
Gagea sp.	6	8	5	8	.	.
Poa bulbosa	11	.	16	8	.	.
Impatiens parviflora	6	.	5	.	13	.
Hygrohypnum luridum d	.	.	.	.	88	78
Bryum pseudotriquetrum d	.	.	.	.	100	44
Cortusa matthioli	.	.	.	.	88	.

TABLE 6. Continued.

Syntaxonomic unit	1	2	3	4	5	6
Aquilegia vicaria	.	.	.	.	63	.
Brachythecium rivulare d	.	.	.	.	38	11
Erianthus ravennae	.	.	.	.	.	22
Parietaria serbica	.	17	5	.	.	.
Callipeltis cucullaris	.	17	.	.	.	.
Taraxacum sp.	.	8	5	.	.	.
Geranium divaricatum	.	.	11	17	.	.
Bromus tectorum	.	.	5	8	.	.
Poa trivialis	.	.	5	8	.	.
Bryum argenteum d	.	.	11	.	.	.
Origanum tythanthum	.	.	11	.	.	.
Sanguisorba alpina	.	.	11	.	.	.
Pseudosedum condensatum	.	.	11	.	.	.
Drepanocaryum sp.	6	.	.	.	.	.
Festuca valesiaca	6	.	.	.	.	.
Lepyrodiclis stellarioides	6	.	.	.	.	.
Paraquilegia uniflora	6	.	.	.	.	.
Rhodiola pamiroalaica	6	.	.	.	.	.
Silene brahuica	6	.	.	.	.	.
Thalictrum alpinum	6	.	.	.	.	.
Aulacospermum dichotomum	.	8	.	.	.	.
Dasiphora parviflora	.	8	.	.	.	.
Veronica capillipes	.	8	.	.	.	.
Veronica rubrifolia	.	8	.	.	.	.
Artemisia absinthium	.	.	5	.	.	.
Conringia planisiliqua	.	.	5	.	.	.
Galium aparine	.	.	5	.	.	.
Phleum graecum	.	.	5	.	.	.
Silene ladygini	.	.	5	.	.	.
Sisymbrium loeselii	.	.	5	.	.	.
Galium spurium	.	.	.	8	.	.
Geranium rotundifolium	.	.	.	8	.	.
Pimpinella peregrina	.	.	.	8	.	.
Calamagrostis dubium	.	.	.	.	.	11
Cicerbita seravshanica	.	.	.	.	.	11
Corydalis kashgarica	.	.	.	.	.	11
Epilobium hirsutum	.	.	.	.	.	11
Gentianopsis vvedensky	.	.	.	.	.	11
Inula macrolepis	.	.	.	.	.	11
Juncus articulatus	.	.	.	.	.	11
Reseda lutea	.	.	.	.	.	11
Rubus caesius c	.	.	.	.	.	11
Schoenus nigricans	.	.	.	.	.	11

phytocoenosis has been recorded in valley bottoms in colline and submontane zones at the relatively low altitudes of 500 to 1,400 m a.s.l. (mean ca. 1,250 m). The association inhabits mainly conglomerates and river gravel sediments, occasionally also limestone rock walls of a relatively low cohesion and acidity of around 6.5 to 7.6 pH. The community develops mainly on southern aspects, however in all cases on overhanging, shaded sites. The approximate mean

inclination is ca. 90°, ranging from 85° to 160° (Fig. 3). The plots of the association occur on wet or humid habitats with dripping water. The *Soncho transcaspici-Adiantetum capilli-veneris* is a species-poor plant community (mean 5 taxa per plot). However, a relatively high cover of the herb layer was recorded due to the abundance of the diagnostic species. The total cover of vascular plants ranged between 40–90%, with a mean value of approximately 65% (Table 3). Mosses covered less than 15% of the plots. In addition to diagnostic species, *Erianthus ravennae* was the most abundant taxon. In the moss layer, *Hygrohypnum luridum*, *Brachythecium mildeanum* and *Bryum pseudotriquetrum* were common.

6. *Cryptogrammetum stelleri* assoc. nova

Typus relevé: Table 5, rel. 3, holotypus hoc loco

Diagnostic species: *Cryptogramma stelleri*

The phytocoenoses of *Cryptogrammetum stelleri* have been noted on limestone and dolomite with alkaline reaction (pH ca. 7.5). The association occurs most commonly on rock faces with coarse fissures close to waterfalls or within the zone of sprayed or dripping water. The phytocoenosis inhabits relatively high elevations in the alpine zone, within the elevational range of 2,100 to 3,000 m a.s.l. (mean ca. 2,200). The *Cryptogrammetum stelleri* develops on rock faces and the bases of rock walls, often in coarse clefts with the mean inclination value of around 70° and various expositions (Figs. 3, 5). The mean herb cover ranges from 15–65% (mean ca. 45%; Fig. 3). The most abundant and constant angiosperm species within the phytocoenosis were *Aquilegia vicaria* and *Cortusa turkestanica*. The moss cover of 2 to 4 species is on average ca. 20%. The most frequent mosses were *Bryum pseudotriquetrum, Hygrohypnum luridum* and *Brachythecium mildeanum*. All of them occur in very wet places, like mires, stream sides or waterfall vicinities.

Discussion

Position of the described association in relation to other types of rock vegetation in Tajikistan.—Three contributions devoted to chasmophytic vegetation of Tajikistan have been published (Nobis *et al.*, 2013; Nowak *et al.*, 2014a, b), focusing on the vascular plant vegetation of solid rock faces, fissures, rock clefts and ledges of the alpine and subnival zone of the Pamir-Alai Mts. These plant communities have been classified into 21 associations assigned within the *Campanuletalia incanescentis* order. This vegetation type is defined by a considerable share of petrophytic species of the Irano-Turanian distributional type, many of them with narrow, endemic ranges in Middle Asian mountains. The rupicolous fern vegetation differs significantly not only in its vascular species composition and considerable share of fern taxa, but also in greater moss cover. For the *Pentanemenion albertoregelii* suballiance the mean cover of mosses is under 5% and for communities from

Campanulenion lehmannianae even less (Nowak *et al.*, 2014a, b). In studied fern communities (see Table 2) the mean value of moss cover is approximately 15% and moss richness is also significantly higher due to microhabitat features such as humidity and soil amount on relatively coarse rock clefts and crevices. It is commonly known that mosses may have a significant role in constituting fern rock communities in mountains (*e.g.* Ochsner, 1954). As compared to other vegetation types, the petrophytic vegetation of Tajikistan, due to the occurrence of narrowly distributed taxa, is characterised by a high syntaxonomical endemism rate on the association level. In the Pamir-Alai Mts, many described associations of rock fissures are defined by taxa confined to one mountain range (*e.g. Sergietum regelii, Scutellarietum megalodontae*) or by species known to occur in only two mountain ranges (*Violetum majchurensis, Andrachnetum fedtschenkoi* and *Eritrichietum turkestanici*). As opposed to these plant communities, fern associations are composed of taxa with wide distributions, sometimes cosmopolitan in the northern hemisphere; thus no endemic species have been used to define associations. Even species with narrower distribution, such as *Cheilanthes persica* or *Cryptogramma stelleri*, are known from several countries of Middle Asia and northeastern Asia. This cosmopolitan character of the fern communities in Tajikistan is mainly due to the absence of effective biogeographical barriers for ferns and mosses. These constraints for species migrations are broadly accepted as a cause of the high rate of endemism in vascular plants other than ferns (Favarger, 1972; Médail and Verlaque, 1997; Strid, 1993).

Obviously altitude greatly influences habitat conditions, especially temperature, precipitation and UV radiation (Latipova, 1968; Vladimirova, 1968). As was earlier indicated for other chasmophytic vegetation of Tajikistan (Nowak *et al.*, 2014a, b), fern communities of rock walls also possess a considerable elevational amplitude. They are distributed from the colline and submontane through the montane and alpine as well as subnival zones (ca. 500–3,200 m a.s.l.). The elevation of ca. 3,500 m seems to delimit suitable conditions for fern vegetation in Middle Asia, especially when considering the eastern part of Tajikistan, with the Pamirian Plateau and its very dry and harsh microclimate, which is very inhospitable and barely accessible for fern communities.

Species composition, chorology and habitat of Tajik petrophytic fern vegetation.—The relatively high diversity of ecological niches among rock habitats influences the considerable floristic richness and diversity of petrophilous fern vegetation in the Pamir-Alai Mts. Despite the low share of endemics in sampled plots, vascular plants and mosses contribute to characteristic species combinations and distinguish between associations. Detrended correspondence analysis clearly reveals two separate groups of samples (Fig. 4). The three associations on the left part of the diagram occur primarily in dry habitats and often grow on bare rocks with a moderate or high share of mosses. To the right the plots of *Asplenio-Cystopteridetum* are grouped, which grow in shadowed places with intermediate humidity. On the opposite side of the graph, *Soncho-Adiantum capilli-veneris* and *Cryptogrammetum stelleri* inhabit humid or even wet places in the immediate vicinity of

water courses. In plots of these communities, hygrophilous moss species, such as *Amblystegium varium*, *Brachythecium mildeanum*, *B. rivulare*, *Hygrohypnum luridum* and *Bryum pseudotriquetrum* are considerably abundant. Nevertheless *Soncho-Adiantetum* and *Cryptogrammetum stelleri*, despite their proximity, differ significantly in elevational range (Fig. 3). The abundance of water is responsible for diminishing the effect of habitat distinctiveness as known from other types of rush or water vegetation in lowlands (e.g. Nowak *et al.*, 2007; Zalewska-Gałosz *et al.*, 2012). Nevertheless, due to their different elevational range, it is not possible to classify both hygrophilous associations within the same alliance (*Adiantion*). Further surveys are needed in neighbouring mountain ranges (*e.g.* Tian Shan, Karakorum, Hindukush) as well as in ranges of Siberia (Ural, Altay) to determine the relevant alliance for highly elevated alpine communities to include *Cryptogrammetum stelleri*. In addition, the vegetation of springs and alpine mires from the *Montio-Cardaminetea* class has to be thoroughly investigated for a precise classification of *Cryptogrammetum stelleri*, because the association has a high share of moss species regarded in European mountains as diagnostic for spring phytocoenoses (e.g. *Brachythecium rivulare*, *Hygrohypnum luridum* and *Bryum pseudotriquetrum*; Chytrý, 2011; Costa *et al.*, 2012). It seems that, moving from left to right in Fig. 3, the main discriminative factor is increasing habitat humidity. The plots of *Asplenio-Ceterachetum* and *Cheilanthetum persicae* (extreme left of figure 3) are distributed in the lowest areas with the warmest and driest microclimates, whereas the plots of *Asplenio-Cystopteridetum* (on the right) have been found in moderate elevations with considerable amounts of precipitation. It is difficult to determine the habitat characteristics related to the vertical axis. It seems that plots of higher elevations, found on bare rocks with a relatively scarce herb layer and moss cover, are grouped in the upper part of the diagram. Especially if the plots of hygrophilous vegetation in the right section of the diagram are excluded, we can see the elevational distinction between colline and submontane *Cheilanthetum persicae* and alpine *Asplenio-Cystopteridetum* and *Asplenietum* quite clearly (Fig. 4, Tables 3–6).

The fern associations of Tajikistan reveal considerable similarities to those of middle and southern Europe, reported from the Alps, the Carpathians, Iberian Peninsula, the Appenines, the Dynaric Mts, the Balkans and southern Turkey. Many of them are characterised by a relatively rich moss layer, feature a typical composition of ferns and mosses occupying shaded or sunny rock clefts, fissures or ledges (Hein *et al.*, 1998; Hruška, 1987; Lovrić *et al.*, 1995; Oberdorfer, 1977; Sanda *et al.*, 2008; Täuber, 1985). However, the different climatic conditions, especially the increased continentality and the higher elevation of habitats, reduce the species richness and abundance of fern communities in Middle Asia. Even in humidity-dependent associations (*Cryptogrammetum stelleri* and *Soncho-Adiantetum*) the diversity of mosses as well as ferns is much less compared to relevant western-Mediterranean phytocoenoses (e.g. Deil, 1998). But in the fern vegetation of warmer sites as well, the scarcity of species constituting the phytocoenoses is apparent. In

TABLE 7. Main ecological habitat characteristics of six fern-dominated rock plant associations in Tajikistan.

Community	Moss cover	Crevice type	Rock type	Insolation	Main exposition	Altitude
Cheilanthetum persicae	M	M/C	C	H	S,SW,W	L/M
Asplenio-Cystopteridetum fragilis	M	F/M	C/N	M	W,NW,N	M/H
Asplenietum trichomano-rutae-murariae	M	F/M	C/N	M	N,NE,S	M/H
Cryptogrammetum stelleri	H	M/C	C/N	M/L	NW,SE	H
Asplenio-Ceterachetum	H	M/C	C/N	L/M	NW,N	L/M
Soncho transcaspici-Adiantetum capilli-veneris	M	M/C	A/C/N	L	S	L/M

Explanations: Soil amount: M - medium, H - high; Crack type: F - fine, M - medium, C - coarse, L - ledge; Rock type: C - calcareous, N - neutral, A - acidophilous; Insolation: H - high, M - moderate, L - low. Exposition: W - western, S - southern, E - eastern, N - northern; Altitude: H - high, M - medium, L - low

European vegetation (e.g. *Drabo lasiocarpae-Ceterachetum officinarum* [Schneider-Binder, Boşcaiu *et al.* 70] Peia 1978; *Sedo-Ceterachetum* Hruška 1985; *Asplenio-Ceterachetum officinarum* Vives 1964; Hruška, 1987; Sanda *et al.*, 2008; Täuber, 1985) the number of co-occurring or co-dominant species is higher than for *Asplenio-Ceterachetum* plots found in Tajikistan. This association is defined by moss species and main diagnostic taxa almost exclusively. Other vascular species can be regarded as sporadic only. Obviously the species composition in other plant associations is also different. Even if the habitat conditions are comparable, and the vegetation is composed of closely related taxa, due to the considerable geographical distance between the Pamir-Alai and the Mediterranean basin, with many barriers between, there are different representatives of the same genera in phytocoenoses. For instance, instead of *Cheilanthes pteridioides*, *Ch. pulchella* or *Ch. marantae* as known in Europe (Sáenz de Rivas and Rivas-Martinez, 1979), in Tajikistan *Ch. persica* is a dominant taxon on sunny and dry walls and rock faces. Nevertheless, doubtlessly none of the described associations has a very specific floristic constitution or could be regarded as a phytocoenosis endemic to Tajikistan. Considering the ranges of the diagnostic moss and fern taxa, surely most of the associations have wide distributions outside Middle Asia, probably far to the west (*Cheilantetum persicae*, *Soncho-Adiantetum*), or alongside the mountain ridges leading to Central Asia and Tibet (*Cryptogrammetum stelleri*).

Another significant feature influencing the diversity of vegetation seems to be elevational range (Fig. 3, Table 7). The highest distribution zone pertains to *Cryptogrammetum stelleri* and *Asplenio-Cystopteridetum fragilis*. Both associations are typical for alpine landscapes with high mountains. *Cryptogramma stelleri*, especially, occurs in harsh, alpine conditions, although it has also been reported from the lower mountains of northern Russia (Iljin, 1934). *Cheilanthetum persicae*, *Soncho-Adiantetum* and *Asplenio-Ceterachetum* find optimum habitat conditions in lower locations, mainly at colline, submontane and montane elevations. This might be attributed to the habitat preferences of

the main diagnostic species or their close relatives from the Mediterranean area (Hruška, 1987; Lovrić *et al.*, 1995; Sanda *et al.*, 2008; Täuber, 1985).

Conclusions.—The present study has summarised the existing knowledge about the fern-dominated chasmophytic vegetation in the Pamir Alai Mts, especially in their western part, with alpine-type mountains. The survey revealed six plant associations, of which three are described for the first time. The associations are well defined by their floristic composition and habitat features although not composed of narrowly distributed, specialised species. Many of fern-dominated communities are rare in Tajikistan due to unsuitable climatic conditions in harsh alpine and subnival zones or in very dry areas with scarce precipitation. Accordingly, the fern communities inhabit very small plots in shaded rock crevices and clefts. Only sporadically the vegetation patches have been found in manmade habitats such as stone walls. Our study reveals that the main factors influencing the species composition within the researched plots is humidity of the microhabitat and is correlated to the abundance of moss species. Also elevation plays an important role in phytocoenoses differentiation. The rock vegetation with a considerable share of pteridophytes still awaits further investigation, especially in forest, shrub and mire phytocoenoses.

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The Growth Pattern of Ophioglossoid Ferns: A Case Study of *Botrychium lunaria* (L.) Sw.

Natalia Olejnik
Department of Plant Taxonomy, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland, e-mail: nmw.olejnik@gmail.com
Zbigniew Celka
Department of Plant Taxonomy, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland, e-mail: zcelka@amu.edu.pl

Abstract.—The family Ophioglossaceae is one of the oldest fern lineages, characterized by a specific sporophyte structure where each leaf is divided into a fertile (sporophore) and a sterile (trophophore) segment. The aims of this study were to analyze: (1) the growth rate and duration for each developmental stage of the sporophyte of the protected fern *Botrychium lunaria* (L.) Sw., (2) the correlation between sporophore and trophophore size, and (3) the effects of soil conditions and density of other herbaceous plants on the development and number of individuals of *B. lunaria* to suggest some possible methods for its protection. Field research was conducted in a threatened population of this species in alluvial ash forest (*Astrantio-Fraxinetum* Oberd. 1953) at the base of Dziewicza Góra, a wooded hill in western Poland. During leaf emergence, the first and longest stage of development, the leaf developed intensively. Subsequently, its subdivision into the sporophore and trophophore became apparent. Throughout the four subsequent stages of sporophyte development (initial maturation, final maturation, spore release and senescence), the sizes of the trophophore and sporophore were significantly correlated. Additionally, sporophore size was affected by abiotic factors, including the total N and organic C contents of the soil. In patches with a higher cover of the herb layer we observed a smaller number of individuals of *B. lunaria*, so active protection by control of competing plant species seems necessary to ensure the survival of this population. Our results may help to design an effective conservation strategy for this rare and threatened fern in Poland and elsewhere.

Key Words.—*Botrychium lunaria*, reproductive success, sporophore, stages of development, trophophore

One of the oldest fern lineages, the Ophioglossaceae, diverges significantly in morphology from that of other ferns (Smith *et al.*, 2006; Szweykowska and Szweykowski, 2012). The sporophyte usually consists of a short branched underground shoot (rhizome) with roots and 1–2 mature leaves with a long petiole and a leaf blade divided into two parts: a pinnate photosynthetic portion (trophophore) with fan-shaped lobes, and a spore-bearing portion (sporophore) (Wagner, 1990; Judd *et al.*, 2010) with sporangia clusters resembling grape clusters.

In Europe, most species of the genus *Botrychium s.l.* are considered rare, e.g., *B.* (*Botrychium s.s.*) *simplex* E. Hitchc., *B.* (*Botrychium s.s.*) *matricariifolium* Fr., *B.* (*Sceptridium*) *multifidum* (S. G. Gmel.) Rupr. and *B.* (*Botrypus*) *virginianum* (L.) Sw. (Page, 1982; Zarzycki; Jackowiak *et al.*, 2007; Shinohara *et al.* 2013). The assumed scarcity of other species such as *B.* (*Botrychium s.s.*) *lunaria* (L.) Sw. is due to the underestimation of the real number of localities,

because their inconspicuous appearance and long dormancy period make them difficult to find (Johnson-Groh and Lee, 2002).

Botrychium lunaria is a circumboreal species found in all parts of Europe except its southern limits (Hultén and Fries, 1986). It grows primarily in sandy and *Nardus*-dominated grasslands, and pioneer communities, like alpine grasslands, especially on calcareous soils (Dostál, 1984; Austrheim and Olsson, 1999; Muller, 1999; Piękoś-Mirkowa and Mirek, 2002). It is also a component of *Molinia* meadows protected in Europe within the Natura 2000 network (Council Directive 92/43/EEC). This species is easily outcompeted by other species when the plant cover becomes dense (Chadde and Kudray, 2001).

Previous ecological studies of *Botrychium* ferns have focused primarily on their relationships with fungi. For example, new fungal taxa of the genus *Glomus* Tul. & C. Tul. have been described in association with the underground developmental stages of *B. crenulatum* W. H. Wagner and *B. lanceolatum* Rupr. (Winther and Friedman, 2007). An ultrastructural study of the *Glomus*-infected rhizome cells of *B. virginianum* (Kovács *et al.*, 2003) was complemented with a molecular analysis (Kovács *et al.*, 2007). Some other studies concerned the ecology and genetic variability of populations of *B. pumicola* Coville ex Underw. (Camacho and Liston, 2001), *B. gallicomontanum* Farrar & Johnson-Groh and B. *mormo* W. H. Wagner (Johnson-Groh and Lee, 2002) and the impact of herbivores and climatic conditions on population characteristics (Mesipuu *et al.*, 2009).

There is little published information on effects of soil conditions on sporophyte growth of Ophioglossaceae. Pindel and Pindel (2007) investigated effects of major soil parameters on 40 fern species in southern Poland, including *B. lunaria*. In Chippewa National Forest, Gundale (2002) investigated how the changes in the soil organic horizon caused by the exotic earthworm *Lumbricus rubellus* affect *B. mormo*. More detailed studies of relationships between soil parameters and pteridophyte growth were conducted for leptosporangiate ferns. For example, effects of soil conditions on N, P, K, Ca and Mg accumulation in fronds of *Athyrium vidalii* (Fr. et Sav.) Nakai were studied by Uozumi *et al.* (2012), while Roivainen *et al.* (2012) investigated the impact of radioisotopes in the soil on a population of *Dryopteris carthusiana* (Vill.) H. P. Fuchs.

The present study focuses on the growth of the sporophyte generation, which is responsible for photosynthesis and spore production. Each organism has limited resources, which must be properly allocated to ensure growth, survival and reproduction (Cody, 1966). The allocation of resources to a given process (e.g., reproduction) will affect their allocation to other processes (e.g., growth and defense). Through resource allocation, individuals can control their responses to environmental conditions. These responses are genetically programmed and may be independent of external conditions (Stearns, 1992; Karlsson and Mendez, 2005). Determining the relationship between the photosynthetic and spore-bearing parts of the sporophyte may be critical to explain the rarity of these ferns. Information about the growth pattern of the sporophyte, the correlation between sporophore and trophophore size, and the

FIG. 1. Stages of sporophyte development in *Botrychium lunaria*. I – leaf emergence, II – initial maturation, III – final maturation, IV – spore release, V – senescence.

effects of abiotic factors on sporophyte development is required to design successful conservation strategies for Ophioglossaceae.

The aims of this study were to analyze: (1) the growth rate and duration for each developmental stage of the sporophyte from three subpopulations of *B. lunaria*, (2) the correlation between sporophore and trophophore size, and (3) the effects of soil conditions and density of other herbaceous plants on the development and number of individuals of this vulnerable species in Poland (Zarzycki and Szeląg, 2006) to suggest some possible methods of protecting *B. lunaria*.

MATERIALS AND METHODS

Study area.—The development of *Botrychium lunaria* was studied in 2011–2012 at the foothills of Dziewicza Góra (143 m a.s.l.), 10 km northeast of the city of Poznań, where one of the largest known populations of this endangered species in the Wielkopolska region (western Poland) occurs (Jackowiak *et al.*, 2007). The study area encompassed three spatially separated subpopulations in ash forest. The distances between these subpopulations were as follows: subpopulations A and B = 5.5 m; B and C = 5 m; A and C = 10.5 m. The local climatic conditions of this area, compared to other parts of Wielkopolska, are warm and cloudy, but with relatively low rainfall. The mean annual precipitation from 1987 to 2007 was 529.3 mm and the mean annual temperature was 8.6°C (Woś, 1994; Grajewski, 2009).

Sporophyte development.—Five developmental stages were distinguished in *B. lunaria* based on Johnson-Groh and Lee's concept (2002) and personal observations (Fig. 1): leaf emergence (I), initial maturation (II), final maturation (III), spore release (IV) and senescence (V). During the first stage, the emerging leaf takes the shape of a compressed ball. During initial maturation (stage II), the leaf blade is divided into a larger trophophore and a smaller sporophore, and the sporangia are green. During final maturation (stage III), the sporophore is larger than the trophophore, and the sporangia are fully developed. During spore release (IV), the sporangia burst, and the spores are dispersed. During the final stage, senescence (V), the aboveground part withers. During the first developmental stage, in March 2012, 20 individuals with undifferentiated

sporophore and trophophore were measured. For the subsequent stages, 50 individuals were analyzed in a research plot of 150 m^2 (13 from subpopulation A, 14 from subpopulation B and 23 from subpopulation C). In total, we found 84 individuals in 2011 and 63 in 2012. The following characteristics were measured with a caliper (1-mm accuracy): total leaf length, sporophore length (from the point where the leaf divides into 2 parts to the top) and trophophore length (measured from the same point to the leaf tip). The plot was monitored weekly. In 2012, we also counted the number of sporangia. The spore release stage was excluded from the measurements, because the dimensions of the sporophyte did not change after final maturation (stage III). Individuals were measured during optimal development (i.e. when 75% of individuals in the population were in the same stage of development) of most individuals of each stage.

Plant-community analysis.—In 2012, a phytosociological analysis (in a relevé) was performed using the Braun-Blanquet method (Braun-Blanquet, 1964). The nomenclature and systematics of the group were adopted from Matuszkiewicz (2012). The vegetation patches encompassing the three subpopulations (plots of 9 m^2 each) were characterized based on the cover percentages of the herb, shrub and tree layers (Table 1). The studied *B. lunaria* population is situated within alluvial ash forest classified as *Astrantio-Fraxinetum* Oberd. 1953. The stand is dominated by *Fraxinus excelsior*.

Soil analysis.—Three soil samples (about 100 g each) were collected from each subpopulation (two from the fern rhizosphere and one control sample from a place without the studied fern from the same patch of vegetation), i.e. a total of nine soil samples. The sample collection and chemical analyses followed standard methods in accordance with Polish regulations (Ostrowska *et al.*, 1991). The following sample characteristics were determined: total N content by the titration method, available P content by the spectrophotometric method, available K content by atomic emission spectroscopy, pH (in 1 n KCl) by the potentiometric method, and organic C content by Turin's method. The samples were analyzed in the Department of Ecology and Environmental Protection of the Poznań University of Life Sciences.

Statistical analyses.—Distributions of the variables were tested using the Kolmogorov-Smirnov test. Because the results were non-significant (i.e., the distributions did not diverge significantly from a normal distribution), an ANOVA was used for repeated measurements and Student's t-test for dependent variables. The correlations between variables were analyzed using the Pearson product-moment correlation coefficient (r). The level of significance was set at 5%. To determine the relationships between the three soil sample collection plots within the alluvial forest, principal component analysis (PCA) was applied. The correlations between the analyzed features (total leaf length, trophophore length, sporophore length and number of sporangia) and soil parameters (pH, K, P, N, N/K, N/P) were determined using canonical correspondence analysis (ter Braak, 1986). The statistical analyses were conducted using the program Statistica 10.0 PL (by StatSoft and CANOCO).

TABLE 1. Characteristics of the subpopulations, vegetation and soil parameters (means ± SD) in three plots with *Botrychium lunaria*.

Characteristic	Plot A	Plot B	Plot C
	Botrychium lunaria subpopulations		
Number of individuals	13	14	23
Density of individuals 2011/2012 per m^2	2.2/2.2	0.9/1.1	6.7/7.7
Mean leaf length 2011/2012 [cm]	12.65/13.28	8.29/12.65	12.58/13.2
Mean sporophore length 2011/2012 [cm]	6.01/6.36	3.23/4.97	6.06/6.63
Mean trophophore length 2011/2012 [cm]	4.82/4.79	2.67/4.59	4.33/4.45
	Vegetation		
Cover of herb layer [%]	90	70	70
Cover of tree and shrub layer [%]	30	50	30
Dominant species[1]	*Astrantia major* *Aegopodium podagraria*	*Aegopodium podagraria* *Mercurialis perennis*	*Aegopodium podagraria* *Mercurialis perennis*
	Soil parameters		
pH in 1 n KCl	5.25±1.08	5.78±0.38	4.71±0.32
K_2O (mg/kg)	48.5±7.5	34.1±4.8	36.9±9.1
P_2O_5 (mg/kg)	12.8±12.3	9.0±6.9	12.8±2.5
Total N (mg/kg)	382±114.0	346±42.7	262±33.6

Note: [1] cover of each species >60%.

RESULTS

Analysis of the plant community.—The shrub layer of the *B. lunaria* plots consisted mainly of *Daphne mezereum*, *Corylus avellana* and *Padus avium*. The rich herb layer was dominated by patches of *Mercurialis perennis*, *Aegopodium podagraria* and *Astrantia major*. The lowest number of *B. lunaria* individuals was counted in subpopulation A, characterized by the highest cover of *Mercurialis perennis* and *Astrantia major* (90%). The highest number of individuals occurred in subpopulation C, where the herb layer covered 70% of the plot and the dominant species were *Aegopodium podagraria* and *Astrantia major* (Table 1).

Trophophore versus sporophore size.—Leaf development lasted in total 79 days. The major characteristics of subpopulations are compared in Table 1. On average, stage I lasted about 20 days, stage II about 14 days, stage III about 25 days, stage IV about 12 days, and stage V about 8 days. In 2012 (the only year, when stage I was observed), fern sporophytes grew 1.5 mm/day from stage I to stage II. From stage II to stage III, the growth rate was 1.1 mm/day in 2011 and 2.3 mm/day in 2012. The mean length of the trophophore and sporophore differed significantly between stages II-III in 2011 (trophophore $t = 7.6$, $p = 0.01$; sporophore $t = 16.2$, $p < 0.001$; Fig. 2A) and in 2012 (trophophore $t = -7.08$, $p < 0.001$; sporophore $t = -10.10$, $p < 0.001$; Fig. 2B). The mean length of the trophophore and sporophore differed significantly during the final stage of growth (stage III) between 2011 and 2012 ($t = -2.650$, $p = 0.009$).

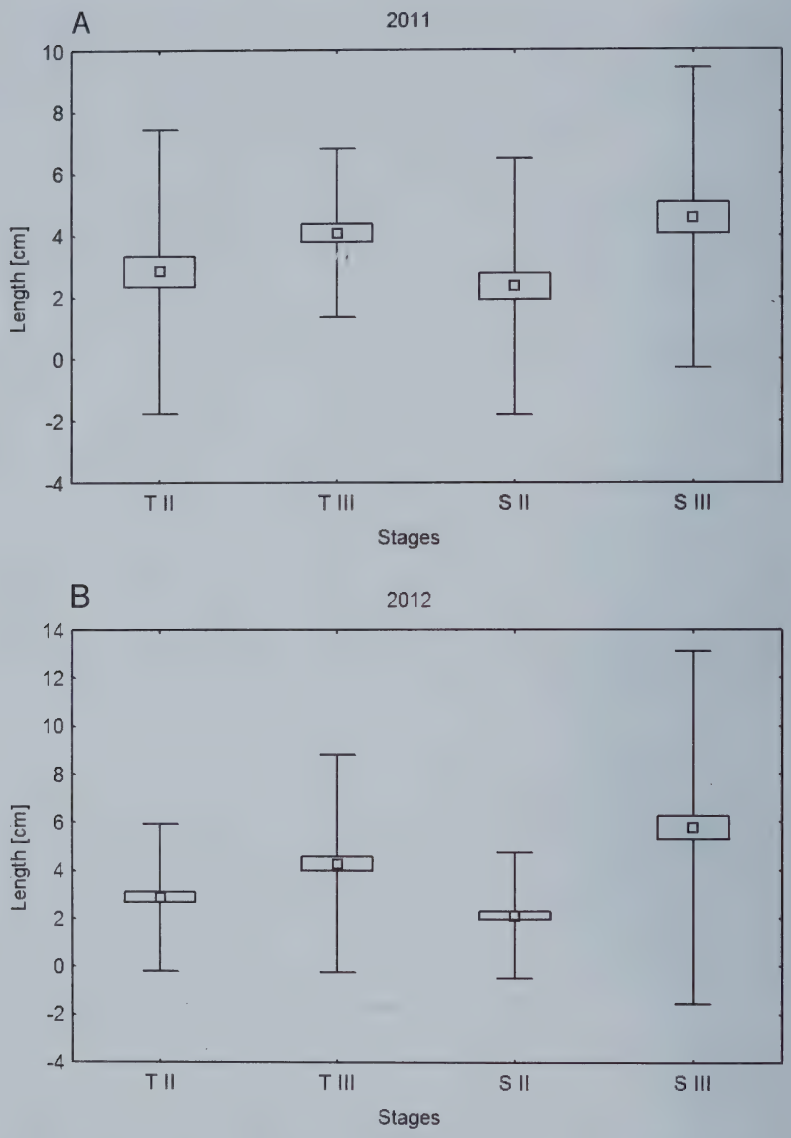

FIG. 2. Trophophore (T) and sporophore (S) length in selected developmental stages of *Botrychium lunaria* in (A) 2011 and (B) 2012: II – initial maturation, III – final maturation, Means with 1 SE and 95% confidence intervals.

During both years of the study, the sizes of the sporophore and trophophore were significantly correlated during stages II–III ($r \geq 0.72$, $p < 0.05$). In 2012, the sporophore length at stage III was significantly correlated with the number of sporangia ($r = 0.81$, $p < 0.05$). In both years, total leaf length and sporophore length of plant individuals at stages II and III were significantly correlated ($r \geq 0.58$, $p < 0.05$).

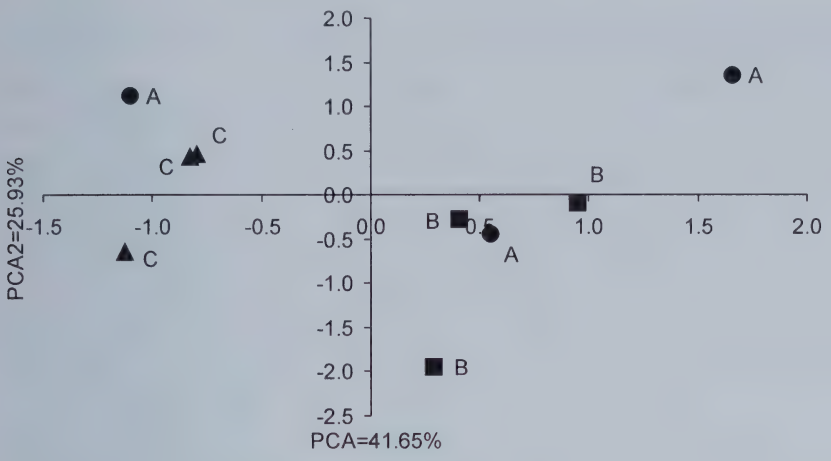

FIG. 3. PCA plot of soil parameters of the three studied subpopulations (A, B, C) of *Botrychium lunaria*

Influence of soil parameters on sporophyte characteristics.—Soil pH in the rhizosphere layer of *Botrychium lunaria* ranged from 4.24 to 6.39. Total N content of the soil samples varied from 225 to 455 mg/kg, whereas organic C content ranged from 2782 to 5088 mg/kg of soil. The studied plots did not differ significantly in the measured soil parameters with exception of the mean $N\text{-}NO_3$ content ($F = 9.917$, $p < 0.05$). The highest soil N content was found in plot A, while the lowest in plot C. However, the scatter diagram for the first two principal components (Fig. 3) showed that the studied three locations did not form a homogeneous group and that soil characteristics were more heterogeneous at site A than at the other two sites. The following parameters were the most strongly correlated with PCA1: total N, N-NH4, organic C and the N/K ratio. K and P contents were the most strongly correlated with PCA2 (Table 2). Canonical correspondence analysis indicated that sporophore length was most strongly correlated with the N-NO3 and N-NH4 contents and the N/K ratio of the soil. Additionally, total leaf length depended on K content, while the number of sporangia depended on P content (Fig. 4).

DISCUSSION

Sporophytes in the family Ophioglossaceae undergo annually several developmental stages, which involve accumulation of reserves followed by reproduction. As a geophyte, *Botrychium lunaria* stores some reserves in the rhizome during the underground mycoheterotrophic phase of the life cycle (Reintal *et al.*, 2010). This probably allows the formation of sporangia already in the first year after emergence of the sporophyte (Klimešová, 2007). On the basis of leaf phenology, Johnson-Groh and Lee (2002) distinguished four developmental stages (leaf emergence, leaf separation, spore release and senescence) in two North American species: *B. gallicomontanum* and

TABLE 2. The correlation coefficients of soil parameters with PCA1 and PCA2. Correlations of $r \geq 0.70$ are marked in bold.

Soil parameter	Correlation coefficient	
	PCA1	PCA2
N	**0.917**	0.151
P	0.400	**0.703**
K	0.012	**0.874**
C (organic)	**0.977**	−0.063
N:P	0.146	**−0.770**
N:K	**0.737**	−0.577
C:N	−0.158	−0.483
$N\text{-}NH_4$	−0.352	0.545
$N\text{-}NO_3$	**0.799**	0.005
pH	0.654	0.025

B. mormo. Similarly, we distinguished in *B. lunaria* the stage of leaf emergence, spore release and senescence, but we subdivided leaf separation into two stages, i.e. initial maturation and final maturation, as frond morphology differs markedly between these two stages. Differences concerned also the timing of all the stages. In the species studied in the USA, individual stages appear earlier (Johnson-Groh and Lee, 2002): *B. gallicomontanum* emerges in early May, when *B. lunaria* is already at the final maturation stage. *Botrychium mormo* emerges even later, in June, which delays also spore release, usually until late August. At this time of the year, *B. lunaria* in Poland has already finished its growing season, which lasts about 11 weeks (from March to June). Most individuals of *B. gallicomontanum* and *B. mormo* complete their seasonal development within about 8 and 13 weeks,

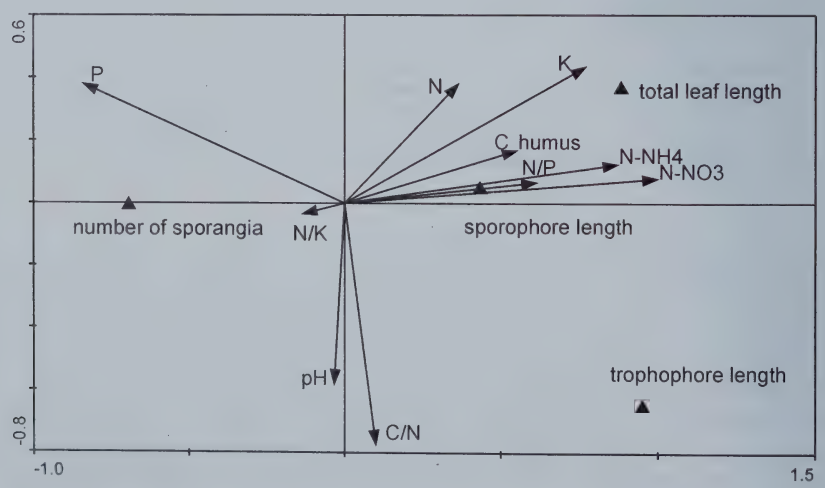

FIG. 4. Canonical correspondence analysis of leaf characteristics of *Botrychium lunaria* (triangles) and soil parameters (arrows).

respectively (Johnson-Groh and Lee, 2002). Duration of individual developmental stages of *B. lunaria* differs from those of North American species. Leaf emergence in *B. lunaria* and *B. mormo* lasts up to about 4 weeks, whereas in *B. gallicomontanum* it takes up to 3 weeks. The separation stage, subdivided in *B. lunaria* into initial maturation and final maturation, is the longest in *B. lunaria* (≤ 5 weeks), but shorter in *B. mormo* (≤ 4 weeks), and *B. gallicomontanum* (≤ 2 weeks). Spore release is the longest developmental stage in *B. mormo* lasting up to 4 weeks (Johnson-Groh and Lee, 2002). These differences are caused by distinct life strategies in various environmental conditions. The length of individual developmental stages of *B. lunaria* is the most similar to that of *B. gallicomontanum*, although the latter grows in prairies, while *B. mormo*, found in maple-basswood forests, has a similar seasonal life span, i.e. annual period of occurrence aboveground (Johnson-Groh and Lee, 2002).

The fitness of individuals and persistence of populations are affected not only by fertility but also by plant size (Harper, 1977). Individuals of *B. lunaria* from the forest population are relatively large. As compared to the population from xerothermic grasslands, the forest plants are on average 4 cm larger (Pindel and Pindel, 2003), and the higher the plant, the more exposed is the sporophore. Spores are dispersed by wind (Peck *et al.*, 1990; Barrington, 1993), so increased plant height facilitates spore dispersal over longer distances. The density of the herb layer seems also important for the development of *B. lunaria*. Our results showed that the largest number of individuals of *B. lunaria* was found in the plot with lowest herb density. Lower densities of *B. australe* were linked to increasing competition for light with other plant species, e.g. *Agrostis capillaris* L. (Sessions and Kelly, 2002). In the case of *B. lunaria*, competing plants had large leaves: *Astrantia major*, *Aegopodium podagraria* and *Mercurialis perennis*.

Environmental conditions strongly affect the distribution and development of pteridophytes in many parts of the world (e.g. Lwanga *et al.*, 1998; Lehmann *et al.*, 2002; Gilman, 2003; Karst *et al.*, 2005; Catapang *et al.*, 2012; Rünk *et al.*, 2012). Detailed research on *Botrychium multifidum* shows a significant effect of environmental conditions and herbivores on its growth pattern (Messipu *et al.*, 2009). For example, higher temperature and precipitation in May and June have a positive effect on sporophyte length in *B. multifidum*, while low precipitation has a negative effect on sporophore growth (Messipu *et al.*, 2009). Roe-Anderson and Southworth (2013) reported that soil moisture during the growing season affects the growth and organic matter content as well as frequency and density of *B. pumicola*. A special role in *B. pumicola* is played by K and N content, which has a positive effect on spore germination and the ability of gametophytes and sporophytes to survive in difficult conditions. A higher concentration of K and N also affects positively symbiotic fungi associated with *Botrychium* species (Roe-Anderson and Southworth, 2013). Moreover, our study provided additional evidence that soil conditions affect the sporophyte development. We have found positive correlations between sporophore size and both N and organic C content of the soil, as well as between total leaf length and K content (see Fig. 4). The analyzed population

of *B. lunaria* grows on black soils (Kasprowicz, 2004), characterized by high N content and a well-developed humus horizon, rich in mineral compounds and organic matter (Mocek *et al.*, 2010). Our observations of the sporophore and results of soil analyses indicate that in such conditions the ramets develop sporophores with numerous sporangia.

The growth pattern of *B. lunaria* did not seem to vary between years and indicated that the emerging leaf stage is always divided into two parts since the very beginning. Sizes of the sporophore and trophophore were significantly correlated in all comparisons, although during initial maturation the trophophore was slightly larger than the sporophore and later the sporophore was extended more quickly and exceeded the trophophore in length during final maturation. Our results demonstrate that the sporophore develops on each leaf, in contrast to other ferns (e.g. *Osmunda regalis*) where some trophophores lack sporophores. Soil conditions and herb cover of large-leaved herbaceous plants (*Mercurialis perennis*, *Aegopodium podagraria* and *Astrantia major*) that compete with the fern, seem to play a major role. Leaf damage was usually caused by trampling of large herbivores such as deer and wild boar. Consequently, future field experiments should investigate how to improve growth conditions of local populations of *B. lunaria* such as by removal of competing plants and by exclusion of herbivores. Our results may help to design an effective conservation strategy for this rare and threatened fern in Poland and elsewhere.

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Propagation and Cryopreservation of *Asplenium scolopendrium* var. *americanum*, the American Hart's-Tongue Fern

Valerie C. Pence
Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, 3400 Vine Street, Cincinnati, OH 45220, USA,
e-mail: valerie.pence@cincinnatizoo.org

Abstract.—*Asplenium scolopendrium* var. *americanum* is a federally-listed fern species that is found in several northern populations in the U.S. and Canada, as well as in three isolated locations in the southern U.S. *In vitro* methods were applied to the germination, propagation, and cryopreservation of this species. Germination of spores was obtained both *in vitro* and on soil, but the production of sporophytes from gametophytes did not begin until over two years after the spores were sown. *In vitro* cultures of both sporophytes and gametophytes were established, but sporophyte production from gametophytes has thus far only been observed on soil. Both *in vitro*-grown gametophytes and sporophytes survived cryopreservation using the encapsulation-dehydration procedure. Preculture on ABA-containing medium increased survival of sporophytes after cryopreservation, but good survival of gametophytes was observed with or without ABA. The use of Green Globular Body-like sporophyte tissue demonstrates the use of this tissue for fern cryopreservation. The success of these protocols demonstrates that *in vitro* methods can be applied to the propagation and preservation of *A. scolopendrium* var. *americanum*, and possibly other fern species with similar growth forms. Propagated plants could assist in restoration of the species and cryopreservation could be used to preserve germplasm of such propagated lines into the future.

Key Words.—conservation, endangered, gametophyte, *in vitro*, sporophyte

The American hart's tongue fern (*Asplenium scolopendrium* var. *americanum* (Fernald) Kartesz & Gandhi) is a federally-listed species found in four states in the U.S. (NY, MI, TN, and AL, where it is also state endangered in MI and TN) and in Ontario, Canada, where it has the status of Sensitive (Center for Plant Conservation, 2010). Most of the plants in the U.S. are found in the northern populations, but a disjunct, apparently relict group of three populations has also been described from sink-hole habitats in southern Tennessee and northern Alabama. Whether in northern or southern populations, this species grows in cool, shaded habitats, on or near dolomitic limestone (U.S. Fish and Wildlife Service, 1993).

The diploid variety of this species (*A. scolopendrium* var. *scolopendrium)* is native to Europe and is common in many areas, while the American hart's tongue fern is tetraploid (Britton, 1953) and is quite rare in North America. There appear to be differences in development between the two, as the gametophytes of the American variety show a greater ability to propagate clonally, suggesting an adaptation to their rockhouse habitat (Testo and Watkins, 2011). Whereas it has been difficult to grow *A. scolopendrium* var. *americanum,* the European variety is easily grown and has been adapted as

a nursery plant, with several horticultural varieties sold both in Europe and in North America (Mickel, 2003).

The American hart's-tongue fern was first described from New York in 1807, while the first occurrence of the southern group was described in Tennessee in 1849 (U.S. Fish and Wildlife, 1993). Two other sites were found in that state, but only one site, in Marion County, remains. However, although 200 plants were reported there in 1898, counts in the past two decades report less than 10 plants remaining, and no spores have been reported from these since the 1980s (Lincicome, 2005). In Alabama, two sites have been reported, in Jackson and Morgan Counties. The Morgan Co. site has been more vigorous, with over 30 plants and some spore production over the past two decades (U.S. Fish and Wildlife, 2012). Spores from this site were germinated and showed some sporophyte production in the mid-1990s (Lincicome, 2005).

The present study was initiated to further investigate propagation methods that could be used for *A. scolopendrium* var. *americanum*. This work focused particularly on whether *in vitro* methods for propagation and cryopreservation could contribute to the conservation of this species, particularly for the southern population of this taxon.

Materials and Methods

One frond with spores and several small leaves of *A. scolopendrium* var. *americanum* were collected from a limestone ledge at the Jackson Co., Alabama, site in July, 2004. Using this material, attempts were made to 1) germinate spores on both soil and medium; and 2) culture leaf tissues, both from the small leaves collected, and from the spore-bearing frond.

Surface Sterilization.—Surface sterilization was done with spores for germination on media, as well as with leaf tissues in preparation for *in vitro* culture. The general protocol used a 1:20 dilution of commercial bleach (final chlorine = 0.26%) plus 0.05% Tween 20 for an indicated length of time, followed by a rinse in sterile, reverse osmosis purified water (RO water). Spores were contained in a packet of folded Whatman No. 1 filter paper during sterilization, while leaf tissues were immersed directly in the sterilant. The only exception to this protocol was used in the field for *in vitro* collecting (IVC) (Pence, 2005), in which the young leaf tissue was sterilized by immersion into 70% ethanol for approximately 20 sec followed by a rinse in sterile RO water.

Media and Growing Conditions.—The basal medium used in these studies consisted of Murashige and Skoog salts with minimal organics (Linsmaier and Skoog, 1965) (MSMO medium, Phytotechnology Laboratories®) plus 88 mM sucrose (Grade 1, Sigma, S5390) and gelled with 2.5 g/L gellan gum (Gelzan™, Caisson G024) (MS medium). In many cases half-strength MS medium (including half-strength sucrose) was used (½ MS medium). Spores were germinated on three media: 1) ½ MS medium, 2) 0.8% agar (Sigma, A1296) medium, and 3) 2K.1N medium, consisting of MS medium plus 25 mg/L KH_2PO_4, (Sigma, P5379), 2 mg/L kinetin (Sigma, K0753), and 0.1 mg/L naphthaleneacetic acid (NAA) (PhytoTechnology Laboratories, N600). Leaf

tissues were cultured on 2K.1N medium. The initial media for spores and tissues from the field, as well as for gametophytes moved from agar plates to higher nutrient medium, also contained 100 mg/L of the fungicide benlate (methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate, Aldrich, 381586-SG) (Ben). All media were adjusted to pH 5.6 ± 0.1 and sterilized by autoclaving at 18–20 psi at 250°C for 15 min. One drop (approximately 0.05 ml) of filter-sterilized (0.2 µ polyethersulfone filter, Nalgene) antibiotic solution (A), consisting of 5 mg/ml cefotaxime (PhytoTechnology Laboratories, C380) and 0.25 mg/ml vancomycin (Sigma, V1130), was also added to the tissues, as described previously (Pence, 2005). For the cryopreservation preculture protocol, tissues were transferred to ½ MS medium with and without 10 µM abscisic acid ((±) ABA, Sigma A1049).

For IVC, medium was distributed into 7 ml scintillation vials, 2.5 ml/vial. For all other tissues, media were distributed into 60 x 15 mm disposable petri plates, approximately 15 ml/plate. One week after collecting, tissues collected by IVC were also transferred to plates of 2K.1N medium. All tissues and spores on media were incubated at 26°C ± 1°C in a 16:8 light:dark cycle under CoolWhite fluorescent bulbs, at approximately 20 µmol/m^2/s, in the CREW incubator room, which houses multiple species. The temperature is similar to the average reported for a number of tissue culture laboratories of 25°C (Davies, 2008)

Spore Germination and Leaf Culture.—Spores were sown both onto sterile soil and sterile medium. For soil germination, spores were sown into soil boxes, which were prepared using well-moistened soilless potting mix (MetroMix) that was autoclaved for 15 minutes at 18 psi, 250°C, and transferred to sterile, clear plastic boxes with lids (Sigma Phytatrays, 10 x 8 x 9 cm), about 3 cm in depth. These were maintained in the laboratory at ambient temperature and fluorescent lighting (approximately 21°C and about 8–10 µmol/m^2/s). For growth on medium, spores were surface sterilized, as described above, for 5 minutes. After rinsing, the packet was opened aseptically, and the spores were blotted onto a sterile medium.

In the case of IVC, surface sterilized young leaf tissue was put into vials of medium in the field. In the case of spore-bearing fronds, after most of the spores were removed in the laboratory for germination, pieces of the leaf were surface sterilized for 5 min and placed onto plates of medium, and incubated as described for *in vitro* cultures above.

Growth in Soil.—Because gametophytes appeared to be more robust on soil, some gametophytes were transferred from agar to soil boxes, to which a few pieces of washed limestone gravel were added to the soil surface. These were kept on the lab bench under conditions described above. Once in soil, gametophytes were allowed to grow and create dense mats within the soil boxes. Watering was done approximately every 2 months with 10–15 ml of RO water/box.

Once sporophytes developed from the gametophytes, they were transferred to similar soil boxes for further growth. Once sterile cultures of sporophytes were established *in vitro*, the resulting clonally propagated sporophytes were acclimatized in the same system and maintained at ambient temperatures and

lighting in the lab, as described above. Survival of both clonal and non-clonal plants in soil appeared to depend on the maintenance of sufficient humidity, which was done by keeping the lids on the boxes closed. In the spring of 2012, three sporophytes were moved from boxes to a terrarium in the CREW greenhouse, with the same soil mixture and pieces of limestone. The terrarium was closed and shaded, and temperature and humidity were measured inside the terrarium using an EasyLog DataLogger (USB Version 5.51, Lascar Electronics Inc.), from June 22, 2012 to July 23, 2012, as well as later, from December 1, 2012 to January 10, 2013.

In Vitro *Culture of Gametophytes and Sporophytes.*—An attempt was made to surface sterilize gametophytes taken from soil boxes, in preparation for culture using the method described above, with 1–5 min in the sodium hypochlorite solution. When this material was then cultured on ½ MS-Ben-A medium in plates, it was either totally bleached (5 min sterilization) or it became contaminated with fungus within a few days (1–2 min sterilization). As a result, an attempt was made to use gametophytes that had been germinated and maintained on agar. Although many of these gametophytes had a small amount of associated bacterial or slow-growing fungal contamination, a sample of gametophytes that appeared clean was transferred to plates of ½ MS-Ben-A medium. These tissues remained clean and established the *in vitro* gametophyte cultures.

After a number of sporophytes had developed from gametophytes in soil, an attempt was also made to initiate sporophyte tissue cultures. Three small sporophytes, approximately 2–3 cm in height, were removed from the gametophyte mat in a soil box and were surface sterilized, as described previously, for 2–3 min, and rinsed twice with sterile RO water. They were then cultured on plates of ½ MS-Ben-A medium, as for the gametophyte cultures. While two of the sporophytes showed no growth, one sporophyte developed green nodular growth from the apical area, but also showed some contamination. Thus, the green nodular tissue was surface sterilized, as described previously, but for only 1 min. During this procedure, the tissue broke apart and some of the nodules were cultured onto the same medium and some were put onto a plate of MS medium with 2 mg/L indoleacetic acid (IAA) and 0.1 mg/L benzylaminopurine (BAP) with no benlate, but antibiotics were added to both media. Tissues on both media grew and established the *in vitro* sporophyte line.

Cryopreservation.—Both gametophytic and sporophytic tissues from *in vitro* cultures were cryopreserved using the encapsulation-dehydration method (Fabre and Dereuddre, 1990), as well as using a method of drying without encapsulation, or "open drying." Fragments of gametophytes approximately 2–3 mm in length were cut from stock cultures, while similar-sized clusters of the green, nodular sporophyte tissue were used. These tissues were transferred to petri plates with preculture medium consisting of ½ MS medium with or without 10 μM ABA (as described above) and incubated for 2–3 days under culture room conditions. For the encapsulation-dehydration procedure, the tissues were then encapsulated in an alginate bead using 3% alginic acid

(PhytoTechnology Laboratories, No. A108) in a solution of MS salts and 0.75 M sucrose and gelled with a 100 mM solution of $CaCl_2$ (Sigma, C3881) in MS salts for 30 minutes. The beads were then transferred to liquid pretreatment medium consisting of MS salts and 0.75 M sucrose and incubated overnight (18–20 hrs), dried for 4 hrs under the air-flow of a laminar flow hood (average flow rate 94 fpm) in open 15 x 90 mm glass petri plates on 2 sheets of Whatman No. 1 filter paper, transferred to 2 ml polypropylene cryovials (Corning), 10–30 dried beads/vial, and plunged into liquid nitrogen (LN) (Fabre and Dereuddre, 1990). Some dried beads were transferred to recovery medium (½ MS medium) without LN exposure as controls. For open drying, after preculture with and without ABA, tissues were transferred directly to filter paper to dry for 4 hrs in the laminar flow hood, as for the encapsulated tissues. After drying, some tissues were transferred to recovery medium as dried controls, while the remaining tissues were transferred to cryovials for rapid freezing in LN. Test vials were removed from LN after 30–60 min, rewarmed at ambient temperature, and transferred to recovery medium to test for survival through LN exposure, while other vials were banked for long-term LN storage in CREW's CryoBioBank™. Survival was measured as pieces of tissue remaining green after two months. In some cases more than one tissue piece was encapsulated in the same alginate bead and these were counted individually; thus, the number of pieces per replicate differed, ranging from 5–16 for the sporophyte tissue, with an average of 9.9 ± 0.4, and 6–24 for the gametophyte tissue, with an average of 12.9 ± 1.2. There were 9 replicates for each sporophyte treatment and 3-6 replicates for the gametophyte treatments. Data were analyzed by ANOVA (Tukey-Kramer) using StatView 5.0.1 (SAS Institute Inc.).

Some beads (containing tissues) were also used to determine moisture content after drying. The dried beads were weighed (W_1), dried at 95°C for 24 hrs and reweighed (W_2). Moisture was calculated on a wet weight basis: $(W_1 - W_2)/W_1$. Moisture determinations were not done with open drying, due to the small amounts of tissue dried, but after drying, the tissues were observed to be brittle and easily broken.

Results

Spore Germination.—Spores that were sown *in vitro* showed germination after one month. Only spores on agar and ½ MS medium germinated, while those on 2K.1N medium did not. However, most spores on ½ MS medium were over-grown by contamination, while a much lower level of contamination was observed on the agar plates. Some spores also germinated on soil, but germination was more frequent in plates. Gametophytes were maintained for approximately 24 months on agar plates with several transfers to fresh agar medium. The gametophytes showed some growth and formed clumps, but they did not produce sporophytes.

Leaf Culture.—Leaf tissue that was cultured did not form shoots or establish propagating cultures. Tissues from the youngest leaves collected and put into

culture using IVC did show some swelling and remained green the longest, but, although the tissues were subcultured onto fresh medium twice in the lab, they did not continue to grow, but eventually browned and died. Tissues from the older, spore-bearing leaf browned and did not show any growth *in vitro*.

Growth in Soil.—Approximately 24 months after spores were sown, some gametophytes were moved from agar to 9 soil boxes. These gametophytes multiplied on the soil and were left undisturbed, except for watering. After 6 months on soil, the first sporophyte was observed from one clump of gametophytes (Fig. 1A). After 3.5 more months, a second sporophyte was observed. Slowly the rate of sporophyte production increased, particularly as the gametophytes formed a dense mat on the surface of the soil (Figs. 1B, 2). As more sporophytes were produced, some were removed and placed in separate soil boxes, but growth of these isolated sporophytes did not appear to be different in rate than those left in the bed of gametophytes. The three sporophytes that were maintained in the terrarium in the greenhouse during the summer of 2012 did not initiate new growth during that time. However, all three survived through the summer and into the next year, even though during that summer they were subjected to temperatures ranging from 24–38°C at 100% RH. During the weeks monitored in the winter, temperatures in the terrarium averaged about 24°C.

In Vitro *Culture of Gametophytes and Sporophytes.*—Tissue culture lines were successfully established from both gametophytes and sporophytes of *A. scolopendrium* var. *americanum*. Attempts to surface sterilize gametophytes growing on soil using 1–2 min exposures to solutions of bleach were unsuccessful, as the tissues quickly became contaminated when cultured on ½ MS medium. Exposure to 5 min of sterilization was lethal. However, initial germination on agar medium, and the transfer of clean-appearing tissues to ½ MS medium containing a fungicide and antibiotics produced some tissues that remained clean-appearing and that continued to grow *in vitro*, with subcultures 2–3 times per year. Tissues grew as an expanded thallus morphology or as tight clusters of tiny gametophytes (Fig. 3).

The nodular tissue that developed from the surface sterilized sporophyte showed growth on both the ½ MS-Ben-A medium and the 2A.1B-Ben-A medium, although it grew more densely on the latter. After two months, this tissue was transferred to ½ MS medium, with and without 10 uM ABA. After two months more, observations indicated that some leaves were growing out of these dense clusters of nodular structures on both media, but that those on ABA showed the most normally developed growth. Tissues from these plates were then moved into culture tubes for maintenance on ½ MS medium (Fig. 4).

Cryopreservation.—Both gametophyte and sporophyte tissues survived drying and cryopreservation using the encapsulation-dehydration method (Fig. 5). Gametophytes showed 75–100% survival when cryopreserved, whether or not they were precultured on ABA. However, sporophyte survival averaged 48% without ABA preculture, while it increased to an average of 84% when treated with ABA. There was no survival of sporophyte tissues through open drying with or without ABA preculture. With gametophytes,

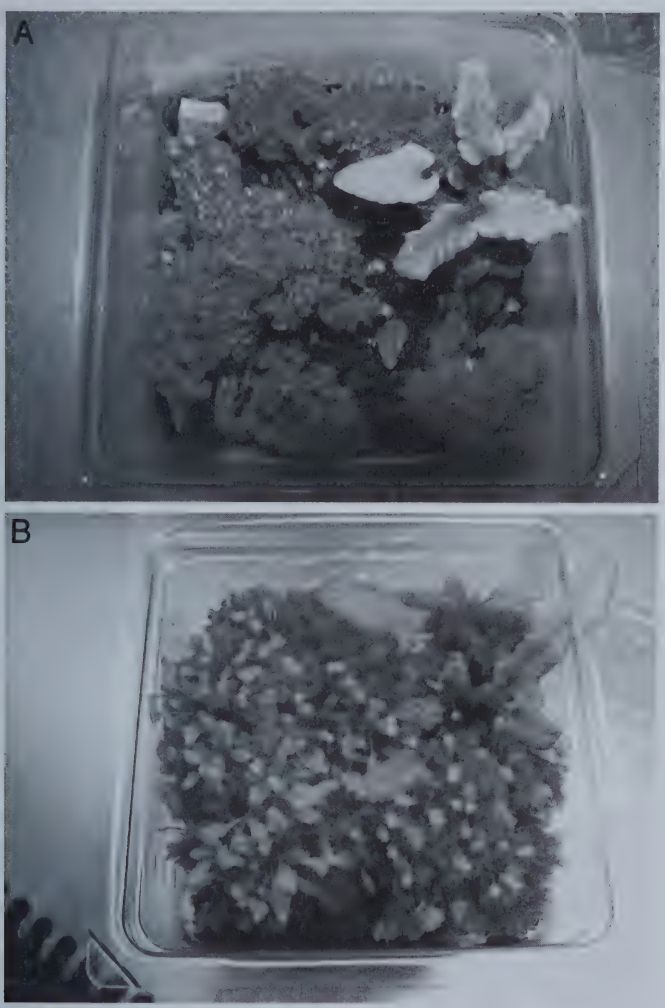

FIG. 1. Development of gametophytes and sporophytes of *A. scolopendrium* var. *americanum* on soil: A. Soil box with dense gametophytes and one sporophyte, about 14 months after gametophytes were moved to soil; B. Same soil box with many sporophytes, about 24 months after move to soil.

only in one attempt out of 4 trials was there some survival through open drying, and only with ABA preculture.

When sporophyte tissues were tested hourly, up to 4 hrs, and moisture loss in the beads was measured, the previous results were supported, in that some sporophyte tissues survived drying up to 4 hrs as well as LN exposure, but that the addition of an ABA preculture increased survival (Fig. 6). Final moisture levels of the alginate beads containing tissues after 4 hrs of drying averaged 22%, and encapsulated tissues survived drying to this level and subsequent freezing. Whereas there was no survival of frozen tissues after 1 hr of drying to

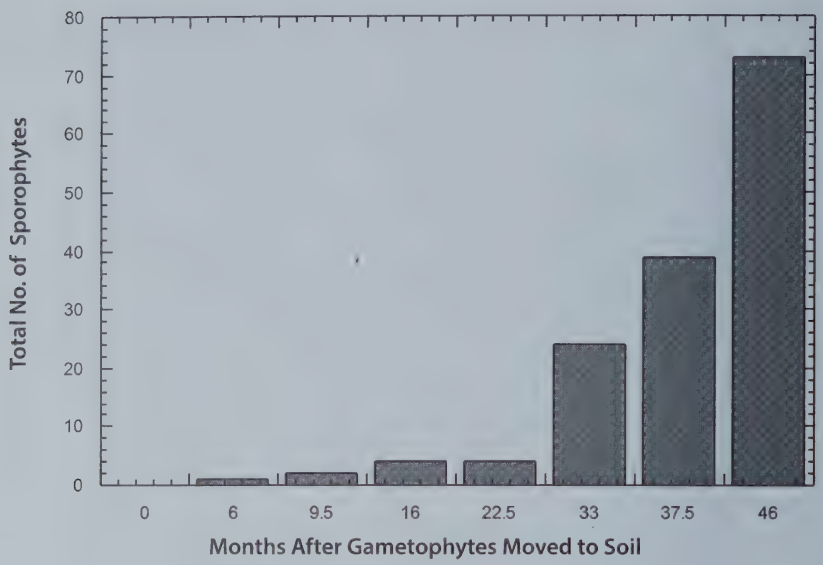

FIG. 2. Increase in the total number of sporophytes in 9 soil boxes containing gametophytes over time, beginning at the time gametophytes were moved from agar plates to the soil boxes.

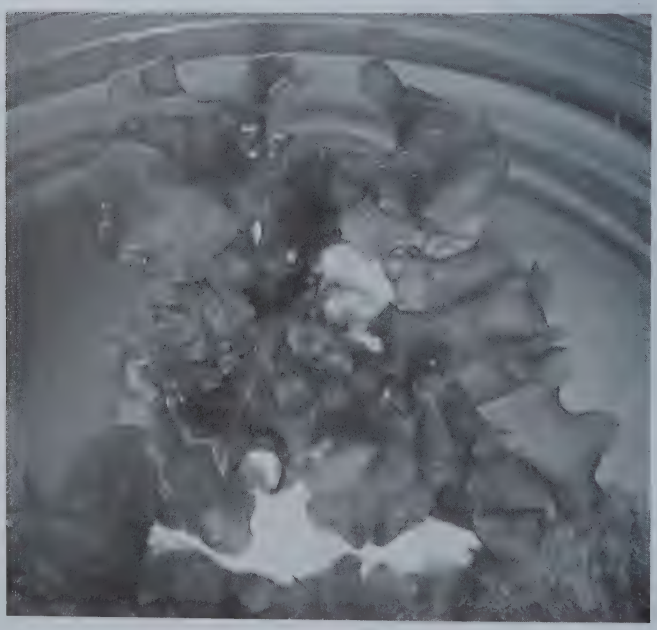

FIG. 3. Gametophytes of *A. scolopendrium* var. *americanum* growing on ½ MS medium *in vitro*, with leafy thallus growth and tight clusters of gametophyte tissue.

FIG. 4. *Asplenium scolopendrium* var. *americanum* sporophyte cultures, with leaves emerging from green nodular clusters.

54% moisture, 2 hrs of drying, to a moisture level of 32% was sufficient for survival through freezing.

DISCUSSION

These results demonstrate propagation and cryostorage protocols for tissues from *A. scolopendrium* var. *americanum*. In this study, initial germination of spores was more prolific on agar than in soil, but later growth was more successful on soil. Gametophytes germinated on soil or moved from agar to soil propagated new gametophytic tissue in the soil boxes, eventually forming a thick gametophytic mat. The first sporophyte appeared from a cluster of

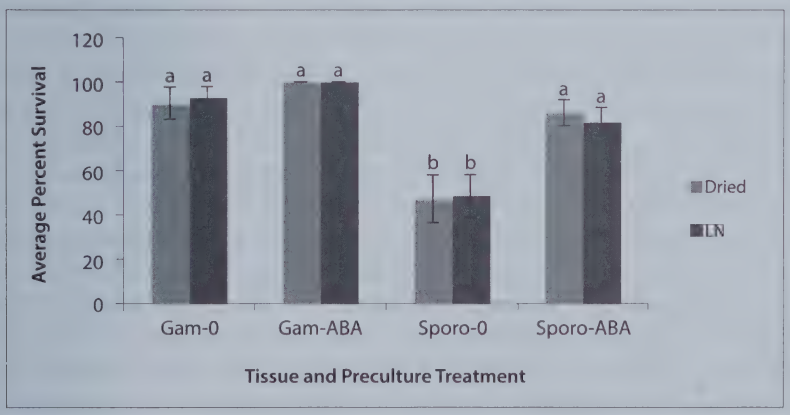

FIG. 5. Percent survival of gametophyte and sporophyte tissues of *A. scolopendrium* var. *americanum* through drying and cryopreservation (LN) using encapsulation dehydration, with a 2–3 day preculture on basal medium with (ABA) and without (O) 10 μM ABA; Gam = gametophyte; Sporo = sporophyte. Different letters indicate significant differences ($p<.05$); bars = standard errors.

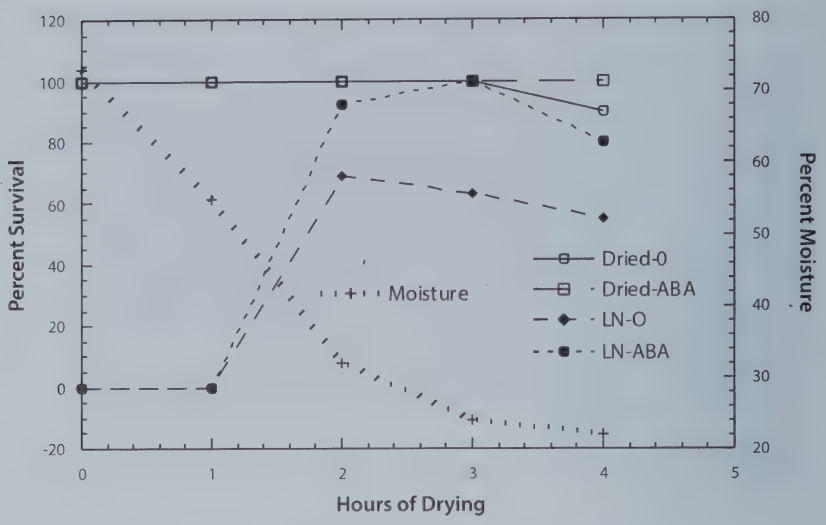

FIG. 6. Survival of encapsulated *A. scolopendrium* var. *americanum* sporophyte tissues, with and without ABA preculture, over the course of 4 hours of drying with and without subsequent LN exposure, and the decrease in moisture content of the alginate encapsulated tissues.

gametophytes at about six months after gametophytes were transferred to soil from agar, but sporophyte production was slow until a thicker mat of gametophytes developed. It is possible that this situation is analogous to conditions observed in a wild, New York population of this species, in which young sporophytes were strongly associated with a dense growth of bryophytes (Cinquemani Kuehn and Leopold, 1993). It was hypothesized that the bryophytes helped protect the young sporophytes from desiccation and the gametophyte mat may serve the same function in the soil boxes.

It is also possible that the temperatures in the laboratory, which are warmer than those recorded in the native habitat, contributed to the slow growth observed in these studies. Temperatures in the Alabama sink-holes have been reported to be relatively constant at 14–15°C (U.S. Fish and Wildlife Service, 2012). Germination of spores of *A. scolopendrium* var. *scolopendrium* has been shown to be slowed at temperatures below 20°C (Pangua et al., 1994). However, work in this laboratory with spores of *Asplenium scolopendrium* var. *scolopendrium* resulted in a much more rapid production of sporophytes from gametophytes under the same soil box and environmental conditions as used for *A. scolopendrium* var. *americanum*, suggesting that these two varieties differ in one or more requirements for either sporophyte formation or growth (unpublished results). This is supported by a recent report by Testo and Watkins (2013) demonstrating that at 20°C the rate of spore germination of *A. scolopendrium* var. *americanum* was significantly lower than that of *A. scolopendrium* var. *scolopendrium*. In addition, they found that sporophyte production from gametophytes of *A. scolopendrium* var. *americanum* was less than 5% after 200 days at 25°C and only increased to about 10% when grown

at 20°C, in contrast to *A. scolopendrium* var. *scolopendrium,* which produced sporophytes at a rate of approximately 70% at both temperatures within that time frame (Testo and Watkins, 2013). These differences suggest that future studies on the effects of temperature on the growth of *in vitro* propagated tissues of *A. scolopendrium* var. *americanum* could be beneficial. In addition, growth was slower and the average number of days needed to produce the first sporophyte was greater in gametophytes of the American variety, compared with the European (Testo and Watkins, 2011). There were fewer male gametophytes and no bisexual gametophytes in *A. scolopendrium* var. *americanum*, compared with *A. scolopendrium* var. *scolopendrium* and the former had a much greater ability to propagate vegetatively than the latter (Testo and Watkins, 2011). These results are consistent with the slow development of sporophytes observed in our studies with *A. scolopendrium* var. *americanum* and may account for the relative rarity of the North American variety compared with the European.

While some mats of gametophytes eventually formed numerous sporophytes, other, similar-appearing mats did not form any sporophytes. Because the formation of the mats appeared to result from clonal production of new gametophyte tissue, it is possible that some boxes contained mats with more genetic diversity than others, depending on the diversity of the initial gametophytes in that box. Wubs, *et al.* (2010) reported that lower sporophyte production occurs from clonal gametophytes than from outcrossing gametophytes in *A. scolopendrium* var. *scolopendrium.* Sporophytes were not observed to arise from *in vitro*-cultured gametophytes in this lab, although gametophytes did not form the dense mats in culture plates that they did in soil. Nutrients are generally depleted in the plates and subculturing is needed before a length of time comparable to that which may be needed for sporophyte formation in soil.

Both gametophytes and sporophytes of this species were successfully initiated into culture. With any tissue, surface sterilization must be timed so that the tissues are sterilized and not killed, but this is particularly difficult with fern gametophytes, which are a single cell in thickness. Short sterilizations were not successful in this study, but germination on agar, followed by selection of clean-appearing material for transfer to medium with a fungicide and antibiotics produced a clean-appearing culture of gametophytes. Further studies with various sterilization times and sterilants, such as sodium dichloroisocyanurate, which has been used with bryophytes (Rowntree and Ramsay, 2005), might also be successful in producing clean gametophyte cultures. In the case of the sporophyte, one of three small sporophytes survived the sterilization and produced propagating tissue.

Sporophytes, whether produced from gametophytes directly or propagated clonally, were reintroduced to soil successfully, but required the maintenance of high humidity for survival. This likely reflects this species' adaptation to moist areas in sinkholes (U.S. Fish and Wildlife Service, 2012). However, the growth of the sporophytes was slow when maintained at ambient conditions in the laboratory, likely reflecting the fact that the lab temperature of about 21°C

is significantly higher than the 14–15°C summer temperatures recorded in their Alabama sinkhole habitat (U. S. Fish and Wildlife, 2012). The three plants that were moved into the greenhouse terrarium survived at least four weeks of frequent temperatures of 100°F in 100% humidity, although they did not show obvious growth during this time, suggesting that this species has the capability of surviving periods of higher temperatures, at least in high humidity.

The ability of tissues of this species to survive encapsulation-dehydration and cryostorage in liquid nitrogen should contribute to the long-term conservation of its germplasm *ex situ*. Survival of gametophytes of the European variety through the encapsulation dehydration procedure was also reported by Mikula *et al.* (2011), but to our knowledge, this is the first report of cryopreservation of the sporophytic form of this species. With the encapsulation dehydration protocol, there was good survival of gametophytic tissue, with or without preculture on ABA, and this is similar to results found with gametophytes of several bryophytes, which do not require ABA preculture to survive encapsulation-dehydration freezing (Pence, 1998). Likewise, the European variety survives freezing by encapsulation-dehydration with or without ABA preculture (Mikula *et al.*, 2011). However, *A. scolopendrium* var. *americanum* sporophytic tissue, while it had some survival without ABA, benefited from ABA preculture to reach levels of survival equivalent to those of the gametophytes. Others have observed that gametophytes may display greater desiccation tolerance than their corresponding sporophytes (Watkins *et al.*, 2007). Previous studies with gametophytes of bryophytes and other species of ferns suggest that while ABA preculture is not needed for survival through encapsulation-dehydration, it is beneficial for inducing survival after open drying (Pence, 1998; 2000). However, there was little or no survival of *A. scolopendrium* var. *americanum* using open drying, with or without ABA preculture.

The growth of *A. scolopendrium* var. *americanum* sporophytes as green nodular clumps in these studies closely resembled the Green Globular Bodies (GGBs) described by Higuchi *et al.* (1987) for *Nephrolepis cordifolia,* a type of growth which is not uncommon in the culture of fern sporophytes and which has been reported in *Asplenium nidum* (Higuchi and Amaki, 1989) and several other species (e.g. Fernandez *et al.*, 1996; Bertrand *et al.*, 1999). Demonstration of the survival of this type of tissue from *A. scolopendrium* var. *americanum* through cryopreservation is significant in that it provides a method for long-term germplasm storage that could be useful for other fern species that show GGB-like sporophytic growth. Very few protocols for the cryopreservation of sporophytes of pteridophytes have been reported, and those have utilized different types of tissues, such as shoot tips in *Selaginella uncinata* (Pence, 2001) and nodes of *Trichomanes punctatum* var. *floridanum* (Pence, 2014). However, the growth of these two species is different than the growth habit of *Asplenium scolopendrium* var. *americanum,* as well as of most ferns, which have leaves arising from a basal area of growth. Isolation of this basal meristem area (the tissue that is needed for cryopreservation) from fern shoot cultures

in vitro, is more difficult and destructive than isolating shoot tips and nodes from either *Selaginella* or *Trichomanes* or from most angiosperm species *in vitro*. Successful cryopreservation of GGB-type tissue provides an alternative tissue that is easy to manipulate and which our results suggest is adaptable for cryostorage, thereby adding another tool for the long-term preservation of fern species that can be induced to produce this morphology *in vitro*.

The Recovery Plan for this species sets a goal to "maintain cultivated sources for the species and provide for long-term maintenance of selected populations in cultivation" (US Fish and Wildlife Service, 1993). The recent 5-year review reiterates the need to "continue developing propagation techniques for the southern populations." In addition there is a need to "evaluate potential for augmenting or reestablishing populations at these sites using sporophyte material produced from collections made at southern states" (U.S. Fish and Wildlife, 2012). Such efforts would also support Target 8 of the Global Strategy for Plant Conservation, "At least 75% of threatened species in *ex situ* collections, preferably in the country of origin, and at least 20% available for recovery and restoration programmes" (Convention on Biological Diversity, 2010). The *in vitro* propagation methods described here should allow for the cultivation of this species *ex situ*. Such cultivation could be maintained long-term, but requires continual input of labor and resources for transfers and medium, particularly if multiple genotypes are initiated into culture and maintained, and adds the risk of loss through contamination. Cryopreservation provides a supplemental tool for maintaining tissues from multiple populations in long-term liquid nitrogen storage, which, after the initial banking, can significantly reduce maintenance costs (Pence, 2011). In addition, initial banking costs for GGB-type tissues will be less than those for banking shoot tips, the method most widely used in banking *in vitro* propagating plant cultures (Reed, 2008). The labor involved in isolating clumps of GGBs is much less than that required for the isolation of shoot tips, and thus the costs of that labor would be less than for many other cryopreservation procedures.

Plants produced through *ex situ* propagation, whether in soil or through tissue culture, are available for restoration projects to increase the number of populations in the south, with the goal of delisting the species (U.S. Fish and Wildlife Service, 1993). This species may also be vulnerable to climate change, since drought has been a factor in reducing numbers in the New York population (Cinquemani Kuehn and Leopold, 1992). Translocation experiments have been reported for the European hart's tongue fern that may be useful in guiding work for restoration or translocation with the American variety (Becker and Becker, 2010), although the more robust nature of the European form suggests that work with the American form may require additional strategies for success. The ability to propagate this species *in vitro* and to cryopreserve propagating lines of gametophytes and sporophytes provides a group of additional tools for maintaining germplasm *ex situ* and producing plants to contribute to research and the long-term management and conservation of this species.

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Studies on Chromosome Numbers and Spore Size in Brazilian *Isoëtes*

Jovani B. Pereira*
Ruhr-Universität Bochum, Department of Evolution and Biodiversity of Plants, 44780 Bochum, Germany; e-mail: Jovani.Pereira@rub.de
Moritz Mittelbach
Ruhr-Universität Bochum, Department of Geobotany, 44780 Bochum, Germany.
e-mail: moritz.mittelbach@rub.de
Paulo H. Labiak
Universidade Federal do Paraná, Depto. de Botânica, C.P. 19031, 81531-980, Curitiba, PR, Brasil;
e-mail: plabiak@ufpr.br

Abstract.—The lycophyte genus *Isoëtes* L. is nearly cosmopolitan, with approximately 350 species, 23 of which occur in Brazil. The lack of adequate distinguishing morphological characters in the leaves and stems, as well as the frequent cases of hybridization and polyploidy, makes the identification of species very difficult. Usually the spores provide the best characters for distinguishing species within *Isoëtes*, but these can vary in size because of polyploidy and hybridization. In this work we studied the variation in size of both mega- and microspores, and the relationship between size and ploidy level. We also present the first chromosome counts for seven species that are endemic to Brazil: *I. goebelii* ($2n = 33$), *I. martii* ($2n = 44$), *I. maxima* ($2n = 22$), *I. organensis* ($2n = 66$), *I. ramboi* ($2n = 44$) *I. smithii* ($2n = 44$), and *I. spannagelii* ($2n = 22$). Our results suggest a correlation between ploidy level and spore size can be useful for the establishment of a preliminary hypothesis on polyploidy and hybridization among Brazilian *Isoëtes*.

Key Words.—Atlantic Rain Forest, hybridization, lycophytes, polyploidy, taxonomy

Hybridization and polyploidization are frequent processes among plants (Leitch and Bennet, 1997; Otto and Whitton, 2000), and both processes, acting together, contribute to the diversification of many of the extant groups. When widespread within a group, hybridization and polyploidy result in a pattern of reticulate evolution that seems to be particularly common among ferns and lycophytes. It is estimated that about 95% of the ferns species have evolved through polyplodization events in their evolutionary history (Haufler, 1987, 2008). The evolutionary complexity of these networks of polyploidy represents a major source of taxonomic confusion, particularly in those groups where the delimitation of species based on morphological characters is very difficult (Small and Hickey, 2001; Watanabe *et al.*, 1996), such is the case in *Isoëtes* L.

The lycophyte genus *Isoëtes* is nearly cosmopolitan, with approximately 350 species (Hickey *et al.*, 2003). *Isoëtes* comprises approximately 23 species in Brazil, 15 of which are narrow endemics to the coastal Atlantic rain forest of southeastern and southern regions (Prado *et al.* 2014). The genus is easily

* Author for correspondence

recognized by leaves with four air chambers and the sunken sporangium on the adaxial base (Moran, 2004). However, the delimitation of *Isoëtes* species is quite difficult because of the few morphological characters that are informative at the species level. Hybridization and polyploidy are also frequent within *Isoëtes* (Taylor and Hickey, 1992), with several polyploid species that show intermediate morphologies between their parents.

Among the main characters that are useful to distinguish the species are those related to spores, and the taxonomy of the genus is almost entirely based on these structures (Hickey, 1986; Pfeiffer, 1922). However, as the spore characters can also be variable, especially in cases where there are events of hybridization and polyploidy, the recognition of primary diploid species, interspecific hybrids, and allopolyploids can provide further information on the genus diversity, as well as on the processes of evolution that have been contributing to its diversification (Taylor *et al.*, 1993).

In this context, analyses of chromosome numbers are a useful tool to understand the taxonomy and the diversification of *Isoëtes*, providing valuable information on: genetic discontinuities within and among species (Windham and Yatskievych, 2003), evolutionary origins of particular lineages (Grusz *et al.*, 2009; Juslén *et al.*, 2011), geographical distribution and ecological tolerances among different cytotypes, and the role of reticulate evolution in the diversification of certain groups (e.g., Gastony, 1988; Juslén *et al.*, 2011; Vamosi and Dickinson, 2006).

Chromosomes counts for *Isoëtes* from the Neotropics, particularly in the mountainous regions from Costa Rica to Argentina, have revealed variation in ploidy levels from $2n = 22$ to $2n = 132$ (Hickey, 1984; Hickey *et al.*, 2003). Studies have also reported the correlation between the ploidy level and the size of spores of either the megaspore or microspore (Luebke and Budke, 2003), or even for both mega- and microspores (Small and Hickey, 2001). Nevertheless, among Brazilian *Isoëtes* chromosome counts have not been conducted, except for the diploid *I. quiririensis* J.B.S. Pereira & Labiak (Pereira and Labiak, 2013). Consequently, the potential value of the spores as a reliable indicator of the ploidy in Brazilian species is still unknown.

The goals of our study were to investigate the ploidy level of Brazilian endemic species of *Isoëtes*, as well as to investigate whether there are correlations between spore size and ploidy level. These data will also serve as a basis for further studies on hybridization and polyploidy, helping to elucidate the importance of these evolutionary processes among the species in Brazil.

Materials and Methods

Material studied.—A total of seven species were analyzed (Table 1). These species were chosen due to the availability of the living materials for chromosome counts. The specimens used for chromosome counting were collected at the type localities for most of the species (except *I. martii* A.Braun ex Kuhn and I. smithii H.P.Fuchs; Table 1). For each population, we collected

TABLE 1. *Isoëtes* species, chromosome number (ploidy level), mean of the spore size with standard deviation (μm), habitat, altitude (m), and locality. $N = 20$ megaspores and 20 microspores per specimen.

Species	Chromosome number (ploidy level)	Megaspore diameter (μm)	Microspore length (μm)	Habitat	Altitude (m)	Collection locality and voucher
I. goebelii U. Weber	33 (3X)	637 ± 39	34 ± 2	Aquatic	2300–2400	Itatiaia, Rio de Janeiro (22°23'01''S; 44°40'11''W); *Pereira 642* (UPCB)
I. martii A. Braun ex Kuhn	44 (4X)	841 ± 25	38 ± 2	Aquatic	2300–2400	Itatiaia, Rio de Janeiro (22°22'36''S; 44°40'12''W); *Pereira 640* (UPCB)
I. maxima Hickey et al.	22 (2X)	617 ± 33	29 ± 1	Aquatic	900–1000	Cambará do Sul, Rio Grande do Sul (29°04'36''S; 49°59'06''W); *Pereira 631* (UPCB)
I. organensis U. Weber	66 (6X)	878 ± 30	42 ± 2	Aquatic	2100–2200	Petrópolis, Rio de Janeiro (22°29'08''S; 43°03'42''W); *Pereira 637* (UPCB)
I. quiririensis J. B. S. Pereira & Labiak	22 (2X)	596 ± 28	31 ± 1	Aquatic	1000–1100	Garuva, Santa Catarina (26°07'36''S; 49°03'13''W); *Pereira 635* (UPCB)
I. smithii H. P. Fuchs	44 (4X)	644 ± 43	33 ± 2	Aquatic	1100–1200	Bom Jardim da Serra, Rio Grande do Sul (28°29'02''S; 49°43'24''W); *Pereira 627* (UPCB)
I. ramboi Herter	44 (4X)	703 ± 34	29 ± 1	Terrestrial	800–900	Cambará do Sul, Rio Grande do Sul (29°04'36''S; 49°59'06''W); *Pereira 630* (UPCB)
I. spannagelii H. P. Fuchs	22 (2X)	538 ± 23	28 ± 2	Terrestrial	1350–1450	Urubici, Santa Catarina (28°08'11''S; 49°38'53''W); *Pereira 626* (UPCB)
I. ulei U. Weber	Unknown	599 ± 46	31 ± 2	Aquatic	2300–2500	Itatiaia, Rio de Janeiro (22°17'08''S; 44°36'47''W); *Condack, 409* (RB)

at least 12 specimens in order to evaluate ploidy level variation among individuals from the same population.

Chromosome counting.—The root tips were fixed in the field using the "Farmer" solution (3 parts 100% ethanol: 1 part glacial acetic acid) and kept at below 15° C. In the laboratory, the fixed roots were maintained below 0° C until examination. Some individuals were collected from wild populations and cultivated *ex situ* for the production of new and additional root tips. For these specimens, the young roots were fixed in Farmer solution and immediately transferred to the freezer to below 0° C. The minimum time of exposure in the fixative was 24 hours.

The roots were hydrolyzed in 5% HCl for 30 minutes, deacidified in 70% ethanol for 3 minutes, and macerated in a drop of a 5% acetocarmine stain. Finally, we added Hoyer's medium in the same proportions as the stain, and squashed following traditional methods (Manton, 1950). The slides were observed using an optical microscope OLYMPUS BX40 with phase contrast. The images were taken using an OLYMPUS DP071.

Spore size.—The diameter of the megaspore and the length of the microspore were measured from the same individuals as used for the chromosome analysis (Table 1), in order to test the correlation between the ploidy level and the spore size. The average size was obtained from 20 mega- or microspores per specimen.

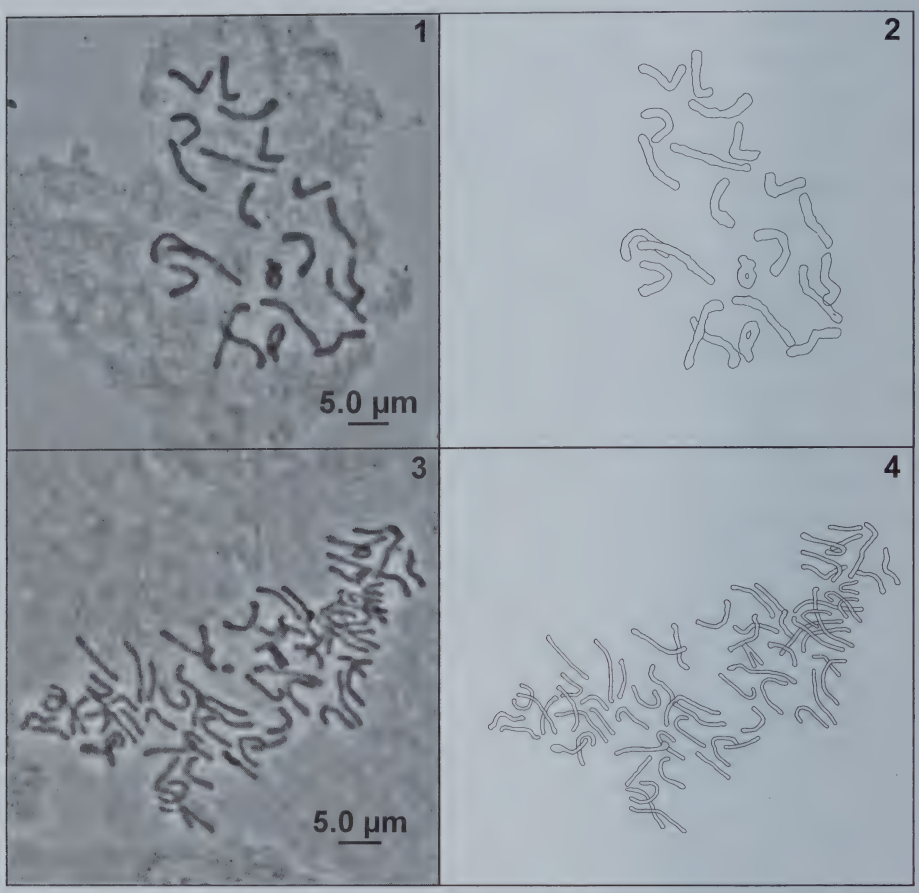
For the microspore measurements, the samples were obtained from dried plant material. The spores, without chemical pretreatment, were mixed with a drop of glycerin on a slide, and immediately observed under an optical microscope OLYMPUS BX40. The images were taken using a camera capture OLYMPUS DP071. Lengths were measured with the program ImageJ 1.46r.

To measure the megaspores, samples were also taken from dried specimens, and imaged using a SEM JEOL model JSM 6360 LV. The megaspore diameters were obtained through the program ImageJ.

Ploidy level and spore size.—To test for a relationship between ploidy level and spore size, we performed a one-way analysis of variance (ANOVA) with a subsequent pairwise Tukey *post-hoc* test for mega- and microspores, separately. Assumptions of homoscedasticity and normality were met using critical values of 0.01 by Levene's test and Bartlett's test, respectively. To test whether diameters of mega- and microspores are correlated, we calculated Pearson's product-moment correlation of averaged spore sizes. All statistical analyses were conducted in R (v. 3.0.2; 2013).

Results and Discussion

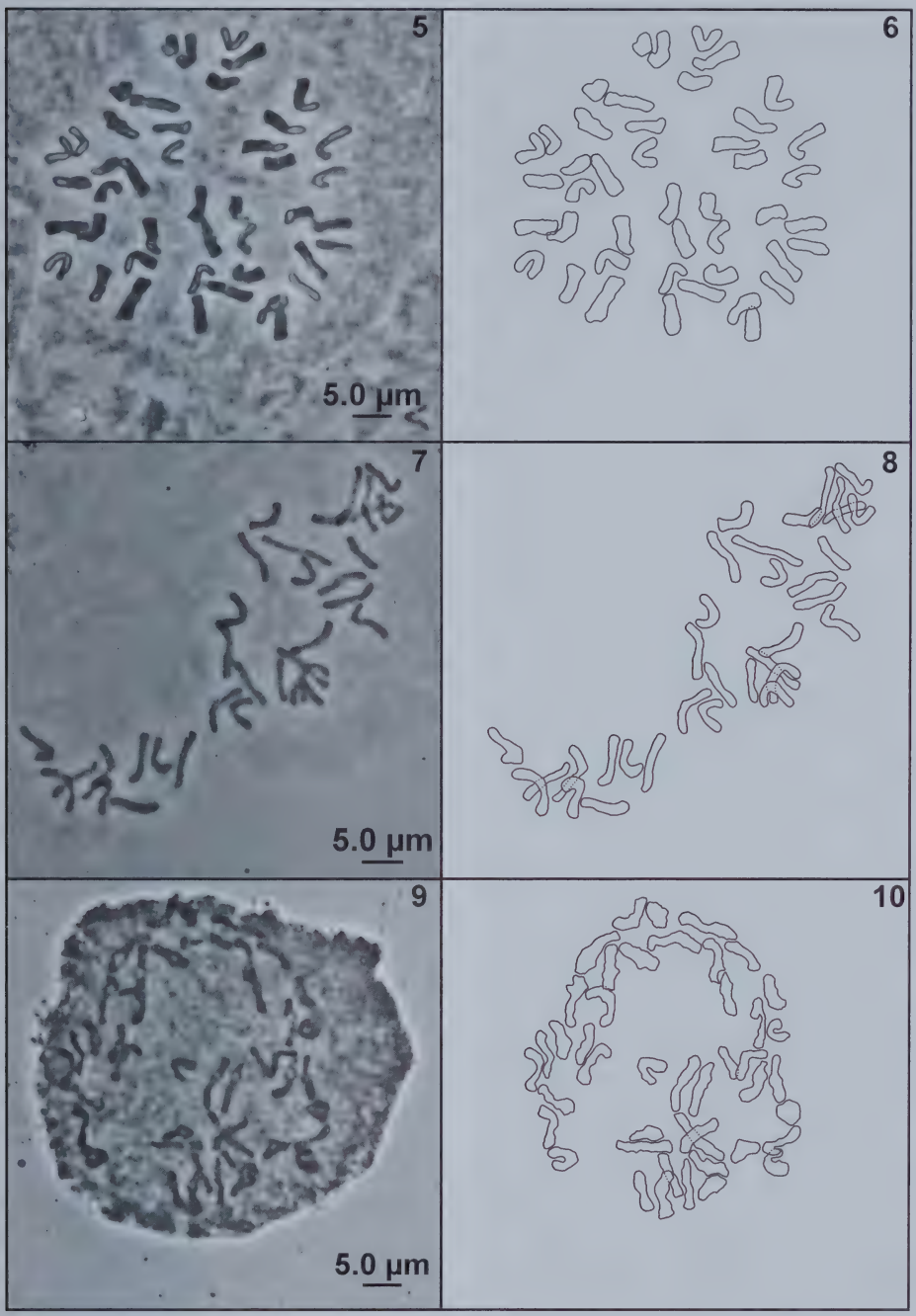
Chromosome counts.—Chromosome counts were obtained for seven of the 15 species of *Isoëtes* that are reported for southeastern and southern Brazil (Figs. 1–16; Table 1). Among them, two are diploid (*I. maxima* and *I. spannagelii*), one is triploid (*I. goebelii*), three are tetraploid (*I. martii, I. ramboi, and I. smithii*), and one is hexaploid (*I. organensis*).



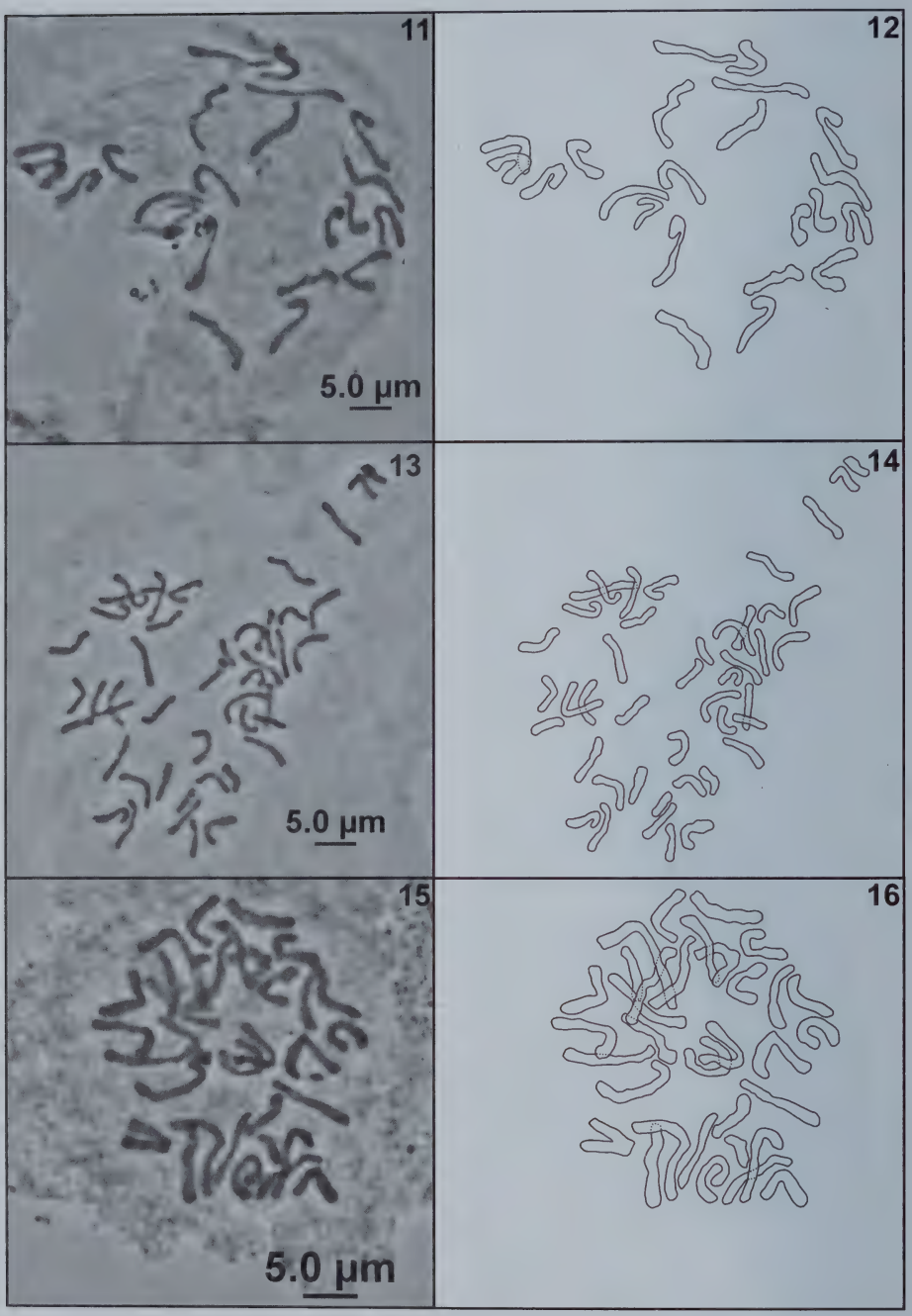
FIGS. 1–4. Photomicrographs and explanatory diagrams of chromosomes at mitosis phase for *Isoëtes maxima* (*Pereira 631*, UPCB) and *I. organensis* (*Pereira 637*, UPCB). 1–2.*Isoëtes maxima*, $2n = 22$. 3–4. *Isoëtes organensis*, $2n = 66$.

The two diploid species, *I. maxima* (Figs. 1–2), and *I. spannagelii* (Figs. 11–12), are endemic to the southern region of Brazil, occurring as aquatic plants on streams and lakes at high elevations. Another diploid species, *I. quiririensis,* has been recorded in similar environments (Pereira and Labiak, 2013), but no evidence for sympatry among these species was found. This suggests that they may represent examples of gradual speciation due to spatial isolation of ancestral populations and genetic divergence, as proposed by Taylor and Hickey (1992) for diploids species from North America.

Isoëtes goebelii is the only triploid that was found among the examined species (Figs. 7–8). This is an aquatic species that grows in permanent lakes and streams at the Itatiaia Highlands (Weber, 1922), with only a few specimens known. Two other aquatic *Isoëtes* are known from the Itatiaia Highlands: *I. martii*, a widespread species in southern Brazil, and *I. ulei*, a locally restricted species. Populations of *I. martii* from Itatiaia were found to be tetraploids



FIGS. 5–8. Photomicrographs and explanatory diagrams of chromosomes at mitosis phase for *Isoëtes martii* (*Pereira 640*, UPCB), *I. goebelii* (*Pereira 642*, UPCB), and *I. ramboi* (*Pereira 630*, UPCB). 5–6. *Isoëtes martii*, $2n = 44$. 7–8. *Isoëtes goebelii*, $2n = 33$. 9–10. *Isoëtes ramboi*, $2n = 44$.



FIGS. 11–16. Photomicrographs and explanatory diagrams of chromosomes at mitosis phase for *Isoëtes spannagelii* (*Pereira 626*, UPCB), *I. smithii* (*Pereira 627*, UPCB), and *Isoëtes* sp. (*Pereira 613*, UPCB). 11–12. *Isoëtes spannagelii*, $2n = 22$. 13–14. *Isoëtes smithii*, $2n = 44$. 15–16. *Isoëtes* sp., $2n = 33$.

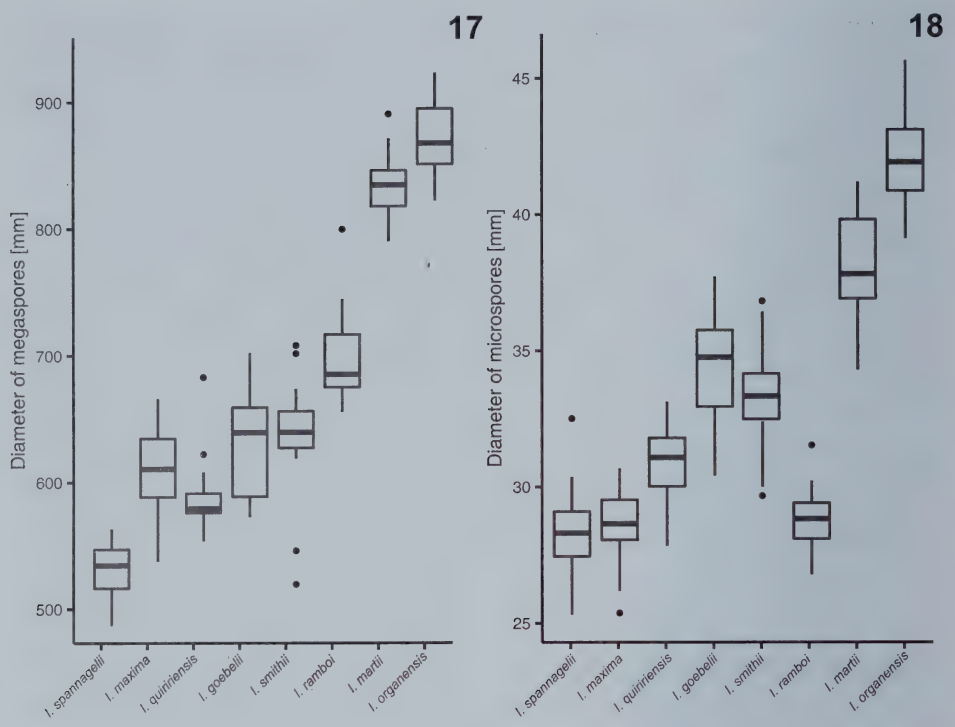
(Figs. 5–6), and it is potentially one of the parental species of *I. goebelli*. As for *I. ulei*, we were not able to obtain chromosome counts due to the scarcity of individuals, but our results on spore sizes suggest that it might be a diploid species (Fig. 19). Whether or not the triploid *I. goebelii* is a result of a hybrid crossing between *I. ulei* and *I. martii*, remains to be proved.

Among populations of *I. smithii* (a tetraploid; Figs. 13–14) and *I. spannagelii* (a diploid), which are growing sympatrically in lakes of Santa Catarina and Rio Grande do Sul states, we also found an indeterminate triploid specimen (*Isoëtes* sp., Figs. 15–16). The length and width of the leaves of this triploid are similar to those of *I. smithii*. However, the specimen was sterile, not allowing us to examine the morphology of the spores for taxonomic identification. This triploid condition suggests that it might represent a new record of an unnamed hybrid between *I. smithii* and *I. spannagelii*, but further studies are needed in order to clarify the origin of this triploid specimen.

Both *I. ramboi* (Figs. 9–10) and *I. smithii* are tetraploids occurring in northeastern Rio Grande do Sul. These two species are very similar morphologically, being distinguished by characters of the velum (in *I. ramboi* it covers almost all of the sporangium, whereas in *I. smithii* it covers up to 50% of the sporangium). Although megaspore morphology has been widely used for species identification (Hickey, 1986), the megaspores show little difference between these two species. As pointed out by Small and Hickey (2001), the tetraploid condition in *Isoëtes* usually occurs by allopolyploidy or, more rarely, by autopolyploidy. Based on the ploidy level of these two species, their sympatric occurrence, and the strong similarities of these two species, it seems likely that they had an allopolyploid origin. This hypothesis, however, remains to be tested within a phylogenetic context.

The highest chromosome number among the Brazilian species was found in *I. organensis* ($2n = 66$), an endemic species that is known from only three populations in the Serra dos Órgãos Mountains of Rio de Janeiro (Figs. 3–4). The chromosome count indicates it is a hexaploid, which is one of the highest ploidy levels ever recorded for a South American species of *Isoëtes*. This hexaploid condition could have arisen through the doubling of the chromosomal number of a hybrid triploid, which, in turn, may have be the result of a cross between a tetraploid and a diploid species (Hickey *et al.*, 2003; Takamiya *et al.*, 1994).

The species examined here, *I. spannagelii*, *I. goebelii*, *I. martii*, *I. smithii*, *I. ramboi* and *I. organensis*, are part of a complex occurring in southeastern and southern South America that have reticulate spores and are taxonomically difficult to separate (Hickey *et al.*, 2009). According to our results, *I. spannagelii*, *I. goebelii*, *I. martii*, *I. smithii*, *I. ramboi* and *I. organensis* constitute a series of different ploidy levels (Table 1), ranging from $2n = 22$ to 66. This suggests that some of these species may have originated by hybridization and auto-allopolyploidy. Similar results were obtained for the complexes of *I. echinospora* in North America (Taylor and Hickey, 1992) and *I. karstenii* in the northern Andes (Small and Hickey, 2001), where similar chromosomal sequences were found.



FIGS. 17–18. Boxplots illustrating the size of mega- and microspores of all sampled *Isoëtes* species. 17. Megaspore. 18. Microspore. ($2n = 22$: *Isoëtes spannagelii*; *I. maxima*; *I. quiririrensis*. $2n = 33$: *Isoëtes goebelii*. $2n = 44$: *Isoëtes smithii*; *I. ramboi*; *I. martii*. $2n = 66$: *Isoëtes organensis*).

Spore size.—The diploids species revealed a variation in the size of the megaspore from 538±23 µm (*I. spannagelii*) to 617±33 µm (*I. maxima*) (Fig. 17; Table 1). As for the microspores, their sizes varied from 28±2 µm, in *I. spannagelii*, to 31±1 µm in *I. quiririensis* (Fig. 18; Table 1). Among the diploids, the smallest mega- and microspores were observed in *I. spannagelii*.

In the triploid *I. goebelii* the sizes of the mega- and microspores were 637±39 µm and 34±2 µm (respectively). Although this species presented a larger microspore in comparison to *I. ramboi* (Table 1; Fig. 18), the megaspore of *I. ramboi* (703±34 µm) is remarkably larger than *I. goebelii* (Table 1; Fig. 17).

The megaspore sizes in the tetraploids ranged from 644±43 µm, in *I. smithii*, to 841±25 µm in *I. martii*, whereas the microspores varied from 29±1 µm (*I. ramboi*) to 38±2 µm (*I. martii*). The microspore of *I. ramboi* seems to be quite smaller in comparison to other tetraploids (Fig. 18).

The largest mega- and microspores were observed in the hexaploid *I. organensis* (878±30 µm, and 42±2 µm, respectively) (Fig. 17–18; Tab. 1). As far as we know, these are among the largest spores recorded for hexaploid species of *Isoëtes*, cf. *I. japonica* A. Braun (467 µm and 30 µm, respetively), *I. coreana* Y. H. Chung & H. K. Choi (447 µm and 32 µm, respectively) (Watanabe

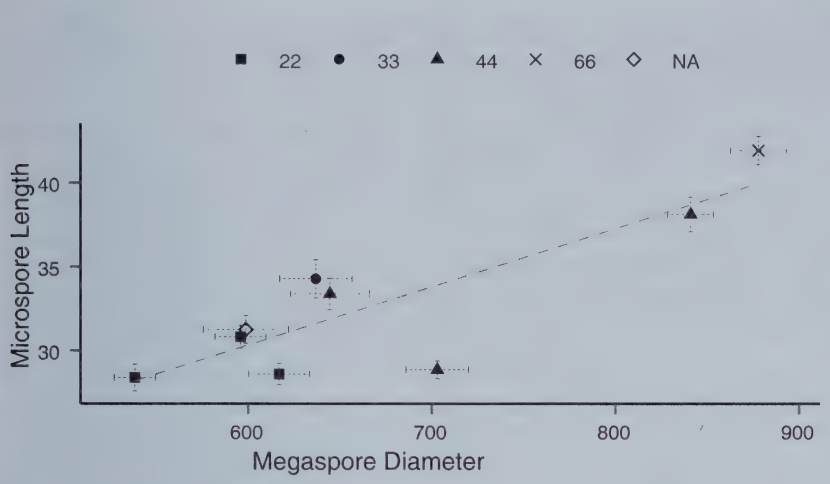


FIG. 19. Scatterplot of average spore size ($N = 20$) of sampled species. Vertical and horizontal bars illustrate standard errors of microspore and megaspore measurements. Shapes are designated according to ploidy level: $2n = 22$ (squares); $2n = 33$ (circles); $2n = 44$ (triangles); $2n = 66$ (X); NA = chromosome number and ploidy unknown of the *Isoëtes ulei* (diamond).

et al. 1996) and *I. orientalis* Hong Liu & Q. F. Wang (420 μm and 22 μm, respectively) (Liu *et al.* 2005), all from East Asia, and *I. chubutiana* Hickey, Macluf & W. C. Taylor (595 μm and 39 μm, respectively) from Argentina and Chile (Hickey et al. 2003). Noteworthy is that the spores are even bigger than the octaploid *I. tennesseensis* Luebke and Budke (753 μm and 36 μm, respectively) from North America (Luebke and Budke 2003).

Ploidy and spore size.—The analysis of variance (ANOVA) revealed that ploidy level is significantly correlated to the size of the mega- (df = 3, F = 124.9, $p<0.001$) and the microspores (df = 3, F = 94.19, $p<0.001$; Table 2). Subsequently, a pairwise Tukey *post-hoc* test supported the hypothesis that mega- and microspore sizes are positively correlated to the ploidy level in all tested ploidy-combinations ($p<0.001$; Table 2), with the only exception being

TABLE 2. Statistical analyses comparing the correlation between ploidy levels and spores sizes in Brazilian *Isoëtes* ($N = 20$ megaspores and 20 microspores per specimen).

Analysis of variance (ANOVA)			
Megaspore	DF = 3	F = 124.9	$p<0.001$
Microspore	DF = 3	F = 94.2	$p<0.001$
Pairwise Tukey *post-hoc* test			
Ploidy level		Microspore	Megaspore
triploid–diploid		$p<0.001$	$p<0.01$
tetraploid–diploid		$p<0.001$	$p<0.001$
hexaploid–diploid		$p<0.001$	$p<0.001$
tetraploid–triploid		$p=0.71$	$p<0.001$
hexaploid–triploid		$p<0.001$	$p<0.001$
hexaploid–tetraploid		$p<0.001$	$p<0.001$

the microspore sizes of the triploid and tetraploids (p=0.71). In addition, the sizes of the mega- and microspore were found to be a significantly correlated to each other ($R^2 = 0.859$, $p < 0.01$).

The correlation between spore size and ploidy has been well documented in the species of the Northern Hemisphere (Cox and Hickey, 1984; Kott and Britton, 1980). In addition, in the *I. karsetii* complex from the Andes, spore sizes were also used as a reliable indicator to predict the ploidy level (Small and Hickey, 2001). However, the ploidy level of species may not always be reflected in sizes of both mega- and microspores, and sometimes only one of the spores can represent the ploidy level in *Isoëtes* reliably (Luebke and Budke, 2003; Watanabe *et al.*, 1996). However, in this study, the sizes of both mega- and microspore were found to each reflect the ploidy level of the species.

It is unsurprising that spore sizes differ according to ploidy level, however, it is noteworthy that spore size is variable for species with the same ploidy level, especially the diploids and tetraploids (Figs. 17–18). We suggest that this variation may be related to the distinct ecological features between different species, and linked to their phylogenetic relationships. These hypotheses remain to be tested in a phylogenetic context.

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Species Richness, Distribution, and Morphological Variation of Lycophytes and Monilophytes in a Semi-arid Region of Mexico

Yarely Pérez-Atilano
Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Hidalgo. Ciudad Universitaria, Km. 4.5 Carretera Pachuca-Tulancingo. Mineral de la Reforma, Hidalgo 42184. México. yarelyatil@gmail.com

Arturo Sánchez-González*
Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Hidalgo. Ciudad Universitaria, Km. 4.5 Carretera Pachuca-Tulancingo. Mineral de la Reforma, Hidalgo 42184. México. arturosg@uaeh.edu.mx

José Daniel Tejero-Díez
Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México Carrera de Biología, Apartado Postal 314, Tlalnepantla, Estado de México 54090. México. tejero@servidor.unam.mx

Abstract.—Most morphological and floristic studies of lycophytes and monilophytes have been conducted in regions with humid and sub-humid climates, leaving dryer regions of the earth virtually unexplored. Arid and semi-arid zones make up about 50% of Mexico's land area; hence, the objectives of this study were (1) to undertake an inventory of fern and lycophyte species present in a region of central Mexico covered mainly by xerophilous vegetation (Valle del Mezquital, Hidalgo); and (2) to analyze patterns of morphological variation of the species found with respect to environmental conditions. Monilophytes and lycophytes were collected in all vegetation types identified within the study area along an elevation gradient between 1200 and 2800 m. For each species, growth substrate, life form and foliar strategy were recorded, and ten morphological characteristics were evaluated in order to determine their variation and correlation with elevation, rainfall, temperature and vegetation type. The data obtained were analyzed using univariate and multivariate techniques. Ten families, 25 genera and 72 species of monilophytes; and one family, one genus and eight species of lycophytes were identified. *Quercus* forest had the highest species richness of lycophytes and monilophytes followed by arid tropical scrub. The most common life forms by substrate were epipetric and terrestrial, the latter represented by chamaephytic, cryptophytic and hemicryptophytic life forms. Most species showed a xeromorphic foliar strategy, as a consequence of prevailing dry conditions. An analysis of morphological leaf characteristics of the species revealed patterns of variation and covariation, primarily related to vegetation type, which are linked to differences in temperature and moisture conditions along the studied elevation gradient. Monilophytes and lycophytes, like other groups of plants, have developed a set of morphological adaptations, which may function together or in various subsets and at different degrees of efficiency to enable plant species to cope with the environmental conditions in their habitats.

Key Words.—ferns, foliar strategy, morphology, PCA, xeric zones.

Monilophytes and lycophytes are seedless vascular plants found in almost every ecosystem, but their highest taxonomic richness is found in humid

* Corresponding author

tropical mountains, where up to 65% of existing species may be concentrated (Page, 1979; Moran, 2008; Hietz, 2010). Interest in lycophytes and monilophytes has increased substantially in recent years, especially in the study of ecological, geographical, palynological, and taxonomic aspects (Moran, 2008; Schuettpelz and Pryer, 2008; Cerón-Carpio *et al.*, 2012; Li *et al.*, 2012; Bogonovich *et al.*, 2013), but because of their worldwide distribution and species richness patterns, most of this research has been conducted in humid and sub-humid climates (Dávila *et al.*, 2002; Anthelme *et al.*, 2011; Cuevas *et al.*, 2013).

Lycophytes and monilophytes are considered to be rare in arid and semi-arid zones (Aldasoro *et al.*, 2004), mainly because of the low efficiency of the stomata of the sporophyte leaf under conditions of water stress (Brodribb and Holbrook, 2004; Brodribb *et al.*, 2009), and the ephemeral, delicate gametophyte phase, which is relegated to humid habitats (Page 2002; Watkins *et al.*, 2007; Hietz, 2010). However, several groups of monilophytes (henceforth ferns) and lycophytes have acquired a wide range of anatomical, physiological and morphological adaptations in their life cycles that enable them to be tolerant or evaders of water stress and to cope with an oligotrophic substrate (Watkins *et al.*, 2007; Mehltreter, 2008; Tejero-Díez, 2009; Hietz, 2010; Watkins and Cardelús, 2012; Pittermann *et al.*, 2013).

The arid and semi-arid ecological zone (*sensu* Toledo and Ordonez, 1998) comprises about 50% of the total land area of Mexico and is considered of great value for its high number of vascular plant species and high percentage of endemic species (both referring to seed plants), and in the case of the ferns of endemic genera, compared to other such sites around the world (Riba, 1993; Mickel and Smith, 2004; Rzedowski, 2006).

The majority of studies on morphological variation in vascular plants have focused on angiosperms and gymnosperms (Yordanov *et al.*, 2003; De Micco and Aronne, 2012). In contrast, for lycophytes and ferns we can only point to the contributions of Kessler (2001), Kessler *et al.* (2007) and Kluge and Kessler (2007). These studies were carried out along elevation and temperature gradients along mountain ranges in South America, but there is no information about this topic in dry environments.

Given the scarcity of morphological and floristic studies of lycophytes and ferns in arid and semiarid regions of the world, the objectives of this study, focusing on adaptations found in these 'adverse' environments, were: 1) to contribute to knowledge of species richness of ferns and lycophytes in dry environments; and 2) to analyze the morphological variation of these species with respect to variations in environmental conditions, specifically elevation, temperature, rainfall and vegetation type.

Materials and Methods

Site description.—The study was carried out in the Valle del Mezquital region, an arid-semiarid area in central Mexico, located in the northwestern part of the state of Hidalgo (Fig. 1). This region encompasses 27 municipalities (county equivalents) and covers about 33.7% of the land area of the state

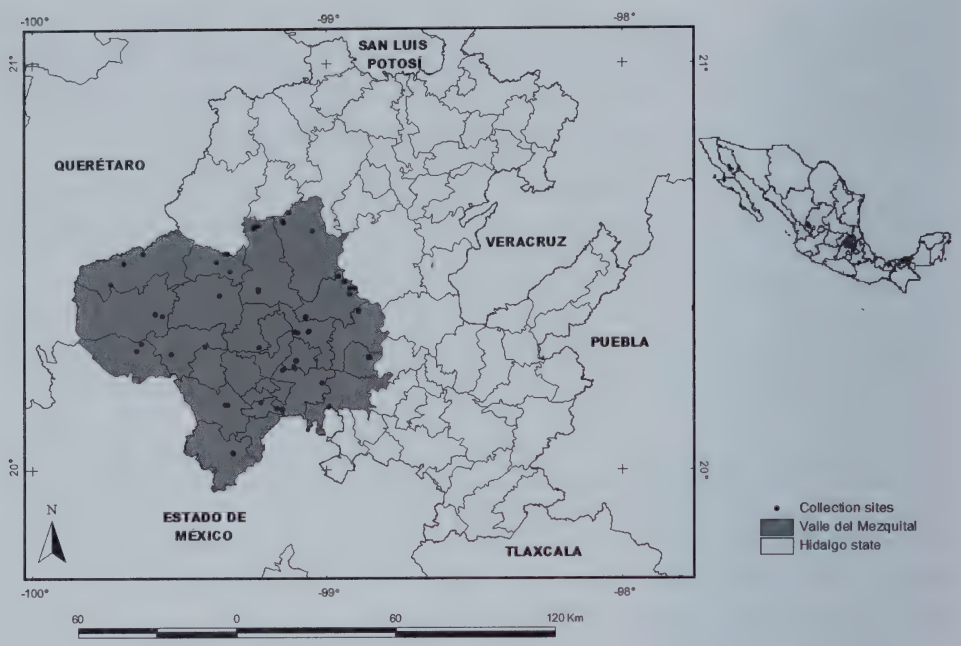

FIG. 1. Study Area in the Valle del Mezquital, Hidalgo State, Mexico.

(Arroyo, 2001). Physiographically, it is located at the continental intersection of the Sierra Madre Oriental and the Trans-Mexican Volcanic Belt (20°24′13" – 20°28′37" N and 98°04′21" – 98°11′14" W), at 1,200 to 3,000 m elevation (González-Quintero, 1968; Arroyo, 2001). The Valle del Mezquital region is surrounded by mountains of differing elevations, which produce a semi-arid climate through rain shadow effects (Cuevas *et al.*, 2013) and ecological gradients, which contribute to the formation of microhabitats. The climate is therefore classified as dry semi-arid; BS1kw(i′)gw" according to the standard Köppen classification modified for Mexico by Garcia (1981). The mean annual temperature ranges between 15.1 and 20.6 °C, and the total annual precipitation between 313 and 525 mm (WorldClim data extracted for collection sites, Hijmans *et al.*, 2005). Ten different vegetation types are present, but arid tropical scrub (according to Rzedowski, 2006) predominates, covering most of the area (González-Quintero, 1968). The vegetation coverage in the Valle del Mezquital is undergoing a high degree of modification. Natural communities are rapidly disappearing, mainly because of overgrazing, selective extraction of species, and changing land use due to agricultural crops and human settlement (Arroyo, 2001).

Fieldwork.—Fieldwork was conducted from 2010 to 2012. Samples of ferns and lycophytes were collected by the traditional method (Lorea and Riba, 1990). Twenty-one municipalities in the Valle del Mezquital, Hidalgo (about 704,498 ha) were explored. The criterion to define vegetation types was qualitative, taking only physiognomy and species dominance into account.

Vegetation type nomenclature was based on the classification of Rzedowski (2006).

Laboratory work.—Collected specimens were identified based on Mickel and Smith (2004), and nomenclature was updated using recent monographs. The plant specimens were deposited in the HGOM herbarium (Autonomous University of the State of Hidalgo, Hidalgo), and duplicates were donated to the MEXU National Herbarium (Institute of Biology, National Autonomous University of Mexico, Mexico City). Additionally, for each fern and lycophyte species the following information was recorded: type of substrate, life-form by substrate according to the classification of Raunkiaer (Mueller and Ellenberg, 1974) and leaf strategy as determined by field observations and a literature review, based on the criteria established by Kessler *et al.* (2007).

The choice of which morphological characteristics to record for evaluation was made on the basis of previous fern studies in the Americas (Kessler *et al.*, 2007; Kluge and Kessler, 2007): (1) laminar dissection (LD), 1 = simple, 2 = pinnate, 3 = bipinnate or more; (2) density of indument – hairs and/or scales leaf present, determined on the adaxial (ADLS) and the abaxial (ABLS) surfaces, classifying each species qualitatively into the following categories: 1 = leaf surface without trichomes, 2 = 1−10% of the leaf surface covered, 3 = 11−25%, 4 = 26−50%, 5 = 51−75%, 6 = 76−100% of the leaf surface covered with indument, (3) stomatal density (SD), determined by counting and averaging the number of stomata in 1 mm^2, taking three (basal, medial and apical) stomatal imprints, made by applying a varnish composed of cellulose acetate and resin (Kessler *et al.*, 2007) for each species; (4) density of venation (DV), the average of the number of veins crossing three 1 cm lines drawn perpendicularly to the rachis at the basal, middle and apical portion of the blade (Uhl and Moosbrugger, 1999); in species with obscured veins, 5% KOH solution was used to clear mesophyll tissue (Kessler *et al.*, 2007); (5) type of rhizome (RT), 1 = erect or decumbent, 2 = short, stout, compact, 3 = short, horizontal or ascending, 4 = short-creeping, horizontal, 5 = long-creeping, horizontal; determined by field observations and species descriptions of Mickel and Smith (2004); (6) leaf morphology (LM), classifying the leaf as 1 = monomorphic or 2 = dimorphic; (7) leaf thickness (LT); 1 = thin, 2 = medium or 3 = thick; (8) indusium (IN), classified as 1 = present, 2 = absent, and (9) hydathodes (HY) classified as 1 = present or 2 = absent for each specimen (Kluge and Kessler, 2007). The lycophytes (*Selaginella* spp.) were not morphologically classified by these criteria because they have microphyllous leaves (Mickel and Smith, 2004).

Processing and data analysis.—Variation in the morphological characteristics was analyzed, calculating the arithmetic mean as the measure of central tendency and the standard deviation (StD) and range as measures of dispersion.

The environmental variables: elevation (m), total annual precipitation (mm), and mean annual temperature (°C) were estimated for each collection site based on the respective geographical coordinates and using the layers (in *.grd format) available from the WorldClim website (1 km spatial resolution) and the

DIVA-GIS 7.3.0.1 program (Hijmans *et al.*, 2001). The magnitude of the correlation between quantitative environmental variables (elevation, rainfall, and temperature) and quantitative morphological characteristics of the specimens was estimated by simple linear correlation (Pearson R). The relationship between categorical variables (HY, IN, LD, LM, LT, RT, VT) and quantitative variables was analyzed with modified Trellis plots (Yeager *et al.*, 2007).

Principal component analysis (PCA) was used to reduce the number of variables, selecting those that explained the highest percentage of total variation in the data and to classify the species into subgroups in the ordination diagram generated from the morphological characteristics of the specimens (Johnson, 2000). The data used in the PCA were the arithmetic mean for each species of the ten morphological characteristics analyzed. All statistical analyses were performed using STATISTICA v7 (StatSoft Inc., 2004).

Results

Fern and lycophyte floristic inventory.—Seventy-two species, one subspecies and eight varieties of ferns (in 25 genera and 10 families) were identified. In addition, eight species of lycophytes were collected, all of which were of the genus *Selaginella* (Appendix). One fern species (*Myriopteris marsupianthes* Fée) and one lycophyte species (*Selaginella nothohybrida* Valdespino) represented new records for Hidalgo State.

The families represented by the greatest number of genera were Pteridaceae with 12 (46.15%), Polypodiaceae with three genera (11.53%), and Dryopteridaceae and Woodsiaceae each with two genera (7.69%; Table 1). The genera represented by the largest number of species were *Myriopteris* (*sensu* Grusz and Windham, 2013) with 15 species, *Selaginella* with eight species, *Pleopeltis* with six species, and *Notholaena* and *Thelypteris*, each with five species. The remaining genera were represented by four or fewer species (Table 1, Appendix).

Of the vegetation types at collection sites in the Valle del Mezquital, *Quercus* forest yielded the highest number of species (25), followed by thorny scrub (22), microphyll scrub (21), and crassicaule scrub (20). The data obtained in the present study and González-Quintero (1978) both indicated that vegetation types above 2,200 m elevation (JS, QF and QS) are found in more favorable conditions (moderate temperature and higher moisture levels) than those at lower altitudes (Table 2, Fig. 3 E–F).

The ferns and lycophytes analyzed together were predominantly epipetric (57.5%) followed by terrestrial (27.5%), while a lower proportion were epiphytic (7.5%) or hydrophytic (Fig. 2a). Three different Raunkiaer life forms were found on terrestrial substrates (Fig. 2b); hemicryptophytes (perennating buds are at the soil surface) = 16.25%, chamaephytes (in ferns, herbaceous plants with perennating buds on aerial branches of less than 50 cm height) = 10%, and cryptophytes (buds are below the soil surface) = 1.25%. The most successful foliar strategy in the study area was xeromorphism (42.5%), while mesomorphic (22.5%), deciduous (18.75%) and poikilohydric (16.25%)

TABLE 1. Number of genera and species of ferns and lycophytes in the study area by family.

Family	Number of genera	Number of species
Pteridaceae	12	44
Polypodiaceae	3	10
Dryopteridaceae	2	3
Woodsiaceae	2	3
Aspleniaceae	1	2
Selaginellaceae	1	8
Anemiaceae	1	2
Thelypteridaceae	1	5
Equisetaceae	1	1
Marsileaceae	1	1
Tectariaceae	1	1

strategies were observed in fewer species (Fig. 2C–D). Mesomorphic and xeromorphic foliar strategies predominated in plants growing on terrestrial substrates (Fig. 2C), xeromorphism in epipetric plants, mesomorphic strategy in marsh plants, and poikilohydric strategy in epiphytes (Fig. 2D).

Morphology of fern species.—The only characteristics to show a wide range of variation, as shown by the measures of central tendency and dispersion, were DV and SD (Table 3).

Among the quantitative morphological variables analyzed, only the combinations DV–SD and ADLS–ABLS showed statistically significant correlations (Spearman, $p < 0.01$), both of them positive (Fig. 3A–D). Correlations among environmental variables were significant between elevation and temperature ($r = -0.43$) and between precipitation and temperature ($r = -0.75$) for the Valle de Mezquital, Hidalgo region (Fig. 3E), but the latter two variables were not correlated with any of the quantitative morphological characteristics. Figure 3F shows the elevation range of each of the vegetation types. Some show a wide range of distribution (CS, MS, RS, TS), while others were found only in the upper range of the elevations studied (for example GF, JS and QF).

In addition, the exploratory analysis of the morphological data by means of the Trellis plots enabled us to identify the following three patterns between categorical and quantitative variables: (1) Species with intermediate and thick lamina have low, intermediate or high values of SD and DV, while species with thin lamina only have low values of SD and DV (Fig. 3A). (2) Species of Pteridaceae have a wide range of values of DV and SD, while the other families have either low to intermediate values (Anemiaceae, Aspleniaceae, Dryopteridaceae, Marsileaceae, Polypodiaceae, Tectariaceae, Thelypteridaceae) or high values (Woodsiaceae) of these characteristics (Fig. 3B). (3) Most species with low values of DV and SD grow in vegetation types or environments with more favorable conditions; higher moisture levels and moderate temperatures (GF, JS, QF and RV; Fig. 3C). In contrast, no significant trend in ADLS or ABLS values by vegetation type or with respect to the other environmental values was observed (Fig. 3D).

TABLE 2. Distribution of lycophytes and monilophytes in study area by vegetation type, and climate conditions. n = number of collection sites.

Code	Vegetation type		Families	Genera	Species	Temperature (°C)*	Precipitation (mm)*	Elevation (m)*	n
10	*Quercus* forest	QF	5	12	25	12–16	700–1,000	2,700–3,000	7
2	Thorny scrub	TS	4	7	22	18	400	1,700–2,000	7
1	Microphyll scrub	MS	6	9	21	18–22	300–600	1,750–2,600	8
4	Crassicaule scrub	CS	5	10	20	16–18	400–600	1,800–2,700	8
6	Riparian vegetation	RV	7	10	16	15–18	360–525	1,200–2,351	5
9	Gallery forest	GF	7	10	11	18	353	2,453–2,655	2
3	Rosette scrub	RS	3	6	11	18–22	300–500	1,750–2,600	2
8	*Quercus* scrub	QS	3	6	8	14–16	500–700	2,200–2,300	1
7	*Juniperus* scrub	JS	4	6	5	12–16	600–800	2,400–3,000	2
5	Secondary vegetation	SV	1	1	1	16	470	2,170	1

*Data from González-Quintero (1968) and WorldClim layers (Hijmans *et al.*, 2005) obtained in present study.

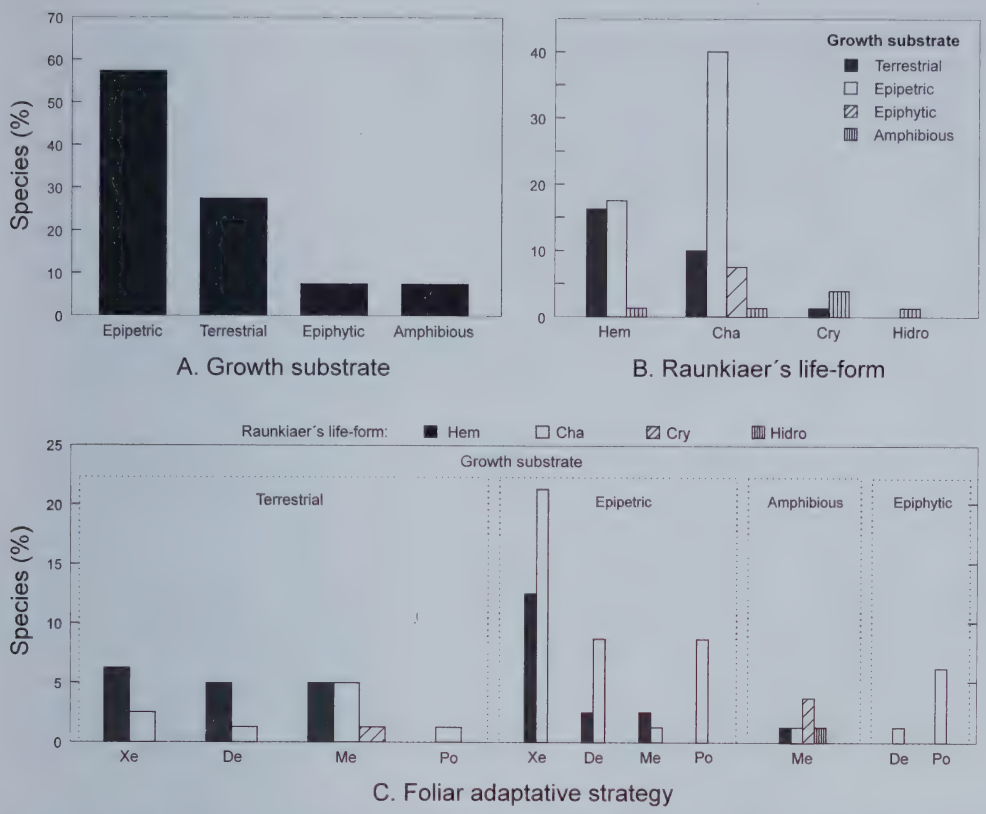

FIG. 2. (A) Growth substrate; (B) Raunkiaer's life-form; (C) Foliar adaptive strategy of ferns and lycophytes in the study area. Life form: Cha = Chamaephyte, Cry = Cryptophyte, Hem = Hemicryptophyte, Hidro = Hydrophyte. Drought-adaptive foliar strategy: Xe = Xeromorphic, De = Deciduous, Me = Mesomorphic, Po = Poikilohydric.

The first two PCA axes explained 46.3% of variation in the morphological characteristics of fern species analyzed (Fig. 4). The variables most correlated with the first principal component were LT, ADLS, ABLS and SD. The characteristics HY, IN and LD are the ones that best represent the variation of the data with respect to the second axis (Table 4).

Most of the species of Pteridaceae that are clustered along the lower end of the ordination diagram (Fig. 4) were collected in desert scrub (Fig. 5). This vegetation type grows in the study area at low and medium elevations, at sites with low rainfall, high temperature and high levels of direct solar radiation (Table 2). Species of Aspleniaceae, Dryopteridaceae, Polypodiaceae and Woodsiaceae are mostly clustered at the upper left corner of the ordination diagram. These species were mainly collected in *Quercus* forests, which grow at higher elevations in the Valle del Mezquital, with average rainfall amounts, moderate temperatures, and in which the ferns are protected from direct sun (Fig. 3, Table 2). Of the environmental variables, only vegetation type showed a statistically significant positive correlation with the first principal component.

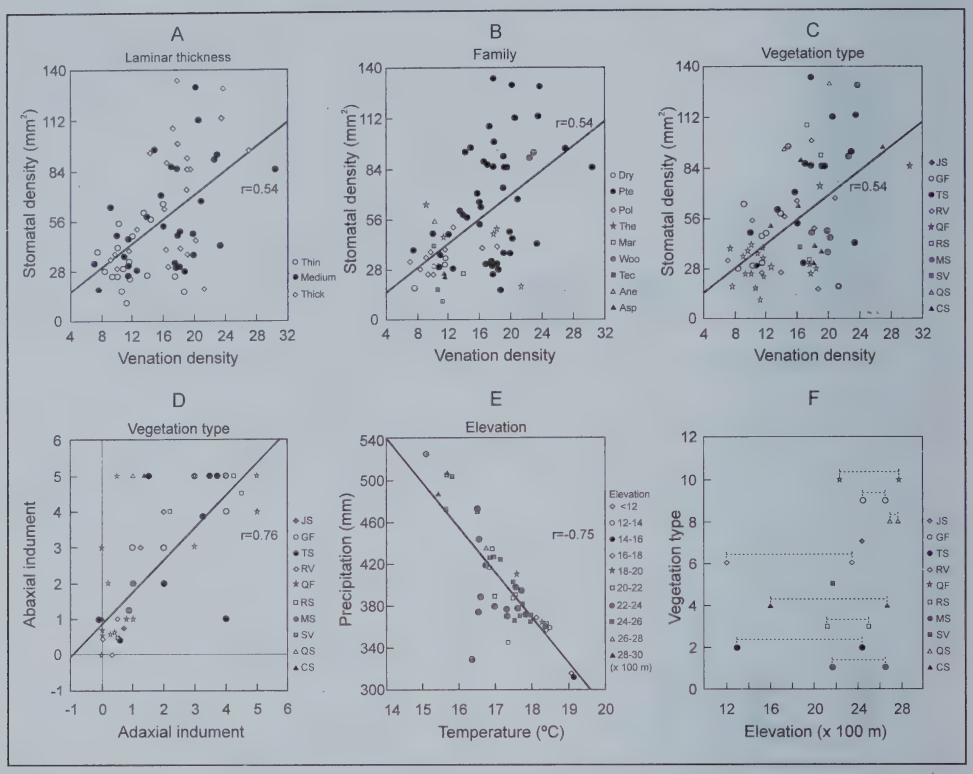

FIG. 3. Linear correlation between stomatal density and vein density with respect to categorical variables: (A) laminar thickness, (B) taxonomic family, (C) vegetation type, (D) linear correlation between adaxial (ADLS) and abaxial indumenta (ABLS) with respect to vegetation type, (E) correlation between temperature and precipitation, with elevation as a categorical variable, and (F) elevation range of vegetation types.

TABLE 3. Morphological characteristics of 72 fern species analyzed.

Characteristic	Units	Minimum value	Maximum value	Range	Arithmetic mean ± StD
RT	1-5	1	5	5	3.19 ± 1.30
LM	1-2	1	2	2	1.04 ± 0.20
LT	1-3	1	3	3	2.08 ± 0.76
HY	1-2	1	2	2	1.04 ± 0.20
IN	1-2	1	2	2	1.68 ± 0.46
LD	1-5	1	5	5	3.37 ± 1.14
ADLS	1-6	1	6	6	2.49 ± 1.62
ABLS	1-6	1	6	6	3.21 ± 1.95
DV	Mean	7.11	30.40	23.29	15.69 ± 4.93
SD	Number/mm^2	10.33	134.50	124.16	55.60 ± 31.05

Characteristics: RT = Rhizome type, LM = Laminar morphology, LT = Laminar thickness, HY = Hydathodes, IN = Indusium, LD = Laminar dissection, ADLS = Adaxial leaf scale and hair density, ABLS = Abaxial leaf scale and hair density, DV = Venation density, SD = Stomatal density. StD = Standard deviation.

TABLE 4. Eigenvalues, explained variance (%), and correlation values (factor loadings) of the first two principal components with environmental and morphological variables.

Component	1	2
Eigenvalue	2.48	2.14
% Cumulative variance	24.88	46.29
Laminar thickness	−0.45	—
Hydathodes	—	−0.40
Indusium	—	0.55
Laminar dissection	—	0.51
Adaxial leaf scale and hair density	−0.44	—
Abaxial leaf scale and hair density	−0.45	—
Stomatal density	−0.44	—
Elevation	0.11	−0.20
Temperature	0.19	−0.97
Precipitation	0.10	0.67
Vegetation type	0.52	−0.23

DISCUSSION

Previous floristic and ecological studies in the Valle del Mezquital have included only vascular seed-bearing plants (González-Quintero, 1968; Ceja-Romero *et al.*, 2010). The results of this study therefore represent a significant contribution to the knowledge of the local lycophyte and monilophyte richness, composition and distribution and in general for arid and semi-arid

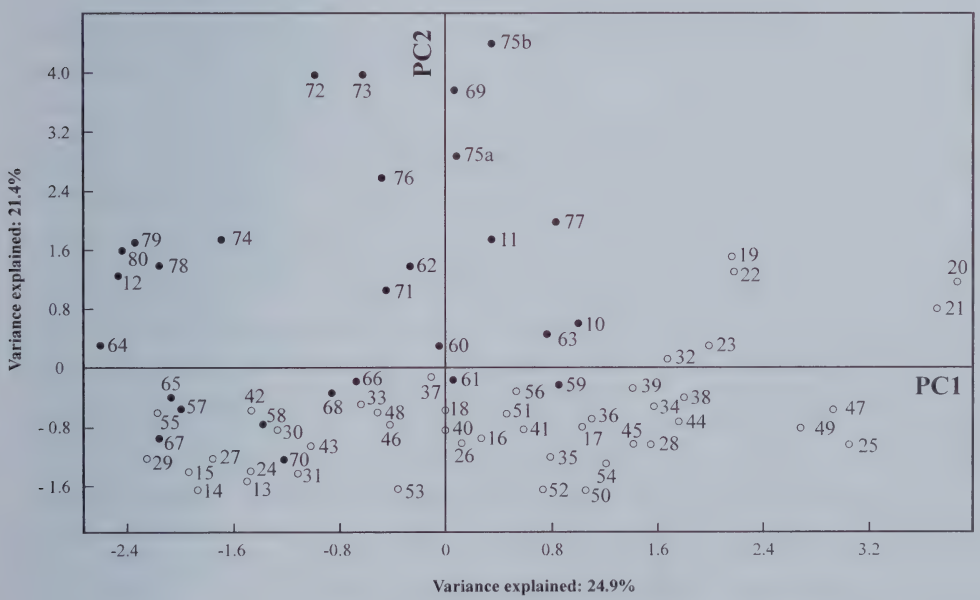

FIG. 4. Ordination plot of morphological characteristics of fern species analyzed and the first two axes or principal components. The species names with their numerical key are included in the Appendix. ○ = Pteridaceae family; • = other families.

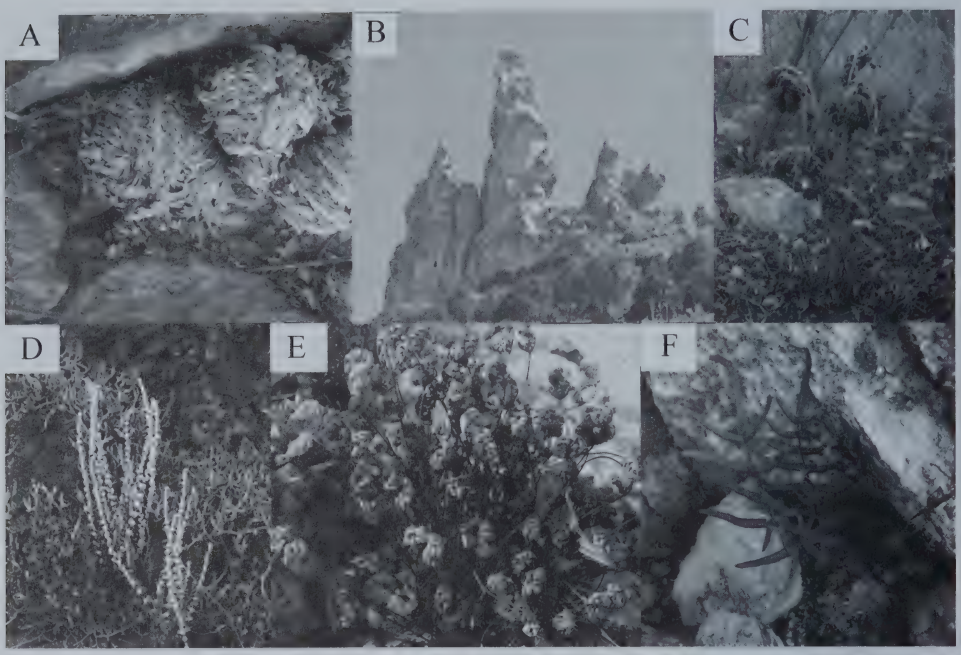

FIG. 5. Species collected and typical landscape in the Valle del Mezquital region, Hidalgo, Mexico: (A) *Selaginella lepidophylla*, (B) "Los Frailes" mountain, (C) *Cheilanthes leucopoda*, (D) *Notholaena affinis*, (E) *Notholaena sulphurea* and (F) *Pellaea atropurpurea*.

regions of Mexico, because we found eighty species of ferns and lycophytes including two new records at the state level: *Myriopteris marsupianthes* and *Selaginella nothohybrida*.

The families with the largest number of genera and species in the Valle del Mezquital: Dryopteridaceae, Polypodiaceae and Pteridaceae, are also the most numerous in other municipalities and regions in the state of Hidalgo, where different environmental conditions, climates and vegetation types prevail (Álvarez-Zúñiga *et al.*, 2012; Pérez-Paredes *et al.*, 2012; Cuevas *et al.*, 2013). This pattern also holds for municipalities or regions of other states of Mexico, such as Mexico State (Tejero-Díez, 2007), Puebla (Cerón-Carpio *et al.*, 2012), Veracruz (Tejero-Díez *et al.*, 2011) and Oaxaca (Tejero-Díez and Mickel, 2004). As such, the results are a reflection of the high level of genus and species richness within these families both in Mexico and globally (Mickel and Smith, 2004; Moran, 2008; Schuettpelz and Pryer, 2008).

The taxonomic inventory includes *Myriopteris*, *Notholaena* and *Pellaea*, genera considered to be representative of the arid tropical scrub of Mexico (Hevly, 1963; Mickel and Smith, 2004; Rzedowski, 2006). Along with *Astrolepis*, *Pleopeltis*, *Polypodium*, *Selaginella* and *Thelypteris*, these genera contained the highest number of species in the study area (51 species; 63.75%). The prevalence of these taxa in the Valle del Mezquital is because most of them have evolved adaptations to survive in dry environments;

strategies that make them either tolerant or evaders of water stress (Schuettpelz and Pryer, 2008; Hietz, 2010; Anthelme *et al.*, 2011).

Vegetation types, substrates and foliar strategies.—Although *Quercus* forest grows only in isolated fragments at sites with high elevation and rough, steep terrain, it was the vegetation type with the highest species and genus richness of ferns and lycophytes. This could be related to the higher moisture levels and more moderate temperatures than in xeric scrub (Rzedowski, 2006). Such conditions are more suited to the eco-physiological requirements of this group of plants (Riba, 1993; Moran, 2008; Brodribb *et al.*, 2009). Moreover, the various associations of desert scrub (crassicaule, thorny and microphyll scrub) identified in the study area had a similar number of genera and species than those of *Quercus* forests, despite that these associations cover much more area of the Valle del Mezquital region (González-Quintero, 1968).

The most common substrates for lycophytes and monilophytes in the various vegetation types are soil, rocks and other plants (Pérez-García *et al.*, 1995). In sub-humid temperate ecosystems, the main growth substrate has been found to be soil, and the predominant life form in this substrate is hemicryptophytic. In humid ecosystems, the hemicryptophytic soil-growing species are joined by those with an erect stem (homeohydric chamaephytes) and the number of epiphytic species often matches or even exceeds the number of terrestrial species (Wolf and Flamenco, 2006; Alvarez-Zúñiga *et al.*, 2012; Pérez-Paredes *et al.*, 2012). However, in drier environments such as the Sahara Desert (Anthelme *et al.*, 2011), the Valle de Tehuacán-Cuicatlán Biosphere Reserve in Puebla (Dávila *et al.*, 2002), the Barranca de Metztitlán Biosphere Reserve in Hidalgo (Cuevas *et al.*, 2013) and the Valle del Mezquital (present study), the highest species richness is found on rock or rock-soil interface, where the main life form is chamaephytic, as the terrestrial environment is generally very homogenous and fluctuating in this ecosystem.

Rocks and the rock-soil interface in arid areas provide special microhabitats, such as cracks, small caves, spaces under boulders and outcrops where environmental conditions are less severe for plants (Hevly, 1963; Aldasoro *et al.*, 2004; Antelme *et al.*, 2011; Cuevas *et al.*, 2013). The Valle del Mezquital is characterized by the presence of xeric scrub, scarcity of forested areas, and rugged terrain with steep slopes, shallow lutite-sandstone soils (which favor moisture retention) and basaltic lithosols (Carrasco-Velázquez *et al.*, 2008), which explains why some 58% of species were collected on rock and only 27% on soil. Moreover, a high representation (42.5%) of drought-adapted xeromorphic species (*sensu* Kessler *et al.*, 2007) was expected and a lower proportion mesomorphic species, as the latter also grow in *Quercus* forests, which occupy only a part of the study area.

Leaf morphology of fern species.—Recently, the high degree of adaptation of ferns to their environmental conditions has been emphasized (Mehltreter, 2008; Hietz, 2010; Cuevas *et al.*, 2013). In dry environments the xeromorphic adaptations of ferns are particularly evident and necessary to offset the lower efficiency of stomatal response to water stress in comparison to that of seed-bearing plants (Brodribb and Holbrook, 2004; Brodribb and McAdam, 2011; Pittermann *et al.*, 2013). In the present study we found that fern species in the

Valle del Mezquital possess one to several morphological adaptations related to habitat and the vegetation type where they grow. These adaptations may be operating together or in various combinations and degrees of efficiency, depending on the environmental conditions and the plasticity of each taxonomic level: species, genera, or family (Hevly, 1963; Kessler *et al.*, 2007; Sreenivasulu *et al.*, 2007; De Micco and Aronne, 2012). In the majority of plant species, water stress is related to various modifications in leaf morphology and anatomy (Sreenivasulu *et al.*, 2007). A trend observed in the present study was that species in mesic environments have low values of LT, SD and DV. In habitats with moderate water deficit, where water balance is more important, the values of these characteristics were intermediate and high (De Micco and Aronne, 2012); in particular Pteridaceae and Woodsiaceae species.

One of the basic assumptions in evolutionary ecology is that closely related species tend to be more similar to each other than to other more distantly related species. Evolutionarily closer taxa may therefore share similarities in characteristics such as morphology and physiology, among others, in their ecological niche (Cooper *et al.*, 2010; Eliosa *et al.*, 2010). The results of this study are generally consistent with the above assumption: in the ordination analysis *Argyrochosma, Astrolepis*, *Bommeria*, *Mildella, Myriopteris, Notholaena* and *Pellaea* species (Pteridaceae), which were collected mainly in arid tropical scrub, shared a set of adaptations such as more variable DV and SD, hairy/scaly indumenta or leaf sclerification (Hevly, 1963; Page, 1979; Tryon and Tryon, 1982; Hemp, 2002; Hietz, 2010). In contrast, species of *Asplenium* (Aspleniaceae), *Dryopteris* (Dryopteridaceae), *Pleopeltis*, *Polypodium* (Polypodiaceae), *Cystopteris* and *Woodsia* (Woodsiaceae) are deciduous or poikilohydric taxa, which evade water stress and have low or intermediate DV and SD, more indument, and are characteristic of forested, shaded and moister environments; and these are phylogenetically more similar to each other (Schuettpelz and Pryer, 2008).

In arid and semi-arid environments, plant survival depends on the species' ability to combine efficiency of structure and function to resist desiccation without suffering permanent damage (De Micco and Aronne, 2012). The results from this study and other recent data (Dávila *et al.*, 2002; Kessler *et al.*, 2007; Watkins *et al.*, 2007; Hietz, 2010; Anthelme *et al.*, 2011; Pittermann *et al.*, 2013) demonstrate that ferns have developed a broad range of leaf adaptations, such as the presence of indument (*Astrolepis*, *Bommeria*, *Myriopteris*, *Notholaena*), increased division (*Adiantum*, *Anemia*, *Argyrochosma*, *Mildella*, *Myriopteris* and *Pellaea*), thickening (*Pleopeltis* and *Polypodium*), or increased density of the veins and/or stomata (Pteridaceae). These adaptations, in different combinations and degrees of efficiency, have enabled ferns to survive successfully in arid and semi-arid environments and to evolve into a more common part of xeric ecosystems than was formerly believed.

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Appendix

Species list of ferns and lycophytes of the Valle del Mezquital, Hidalgo, Mexico

Taxonomic group	Vegetation type	Substrate	Life form	Foliar strategy
Lycopodiophyta				
Selaginellaceae				
1. *Selaginella arsenei* Weath.	MS,RV	Ep	Cha	Po
2. *Selaginella lepidophylla* (Hook. & Grev.) Spring in Martius	CS,TS,RS,RV	Ep	Cha	Po
3. *Selaginella nothohybrida* Valdespino	CS	Ep	Cha	Po
4. *Selaginella pallescens* (C. Presl.) Spring *in* Martius	JS,MS,QS,RV	T	Cha	Po
5. *Selaginella peruviana* (Milde) Hieron.	TS	Ep	Cha	Po
6. *Selaginella reflexa* Underw.	TS	T	Cha	Me
7. *Selaginella rupincola* Underw.	CS,TS,MS,RS	Ep	Cha	Po
8. *Selaginella wrightii* Hieron.	CS,TS,MS,RS	Ep	Cha	Po
Polypodiophyta				
Equisetaceae				
9. *Equisetum* sp.	GF	A	Cry	Me
Anemiaceae				
10. *Anemia adiantifolia* (L.) Sw.	TS,MS	Ep	Hem	Me
11. *Anemia mexicana* var. *mexicana* Klotzsch	TS	Ep	Hem	De
Marsileaceae				
12. *Marsilea mollis* B. L. Rob. & Fernald	CS,RV	A	Hy	

Appendix. Continued.

Taxonomic group	Vegetation type	Substrate	Life form	Foliar strategy
Pteridaceae				
13. *Adiantum andicola* Liebm.	RV	T	Hem	De
14. *Adiantum capillus-veneris* L.	RS,RV	Ep	Cha	De
15. *Adiantum poiretii* Wikstr.	GF,QF,QS	T	Hem	De
16. *Argyrochosma formosa* (Liebm.) Windham	TS,MS,RS	Ep	Cha	De
17. *Argyrochosma incana* (C. Presl) Windham	QS	Ep	Cha	De
18. *Argyrochosma pallens* (Weath. ex R. M. Tryon) Windham	CS,MS,RV	Ep	Cha	De
19. *Astrolepis crassifolia* (T. Moore & Houlston) D. M. Benham & Windham	QF	Ep	Cha	Xe
20. *Astrolepis integerrima* (Hook.) D.M. Benham & Windham	MS	Ep	Cha	Xe
21. *Astrolepis laevis* (M. Martens & Galeotti) Mickel	TS,MS	Ep	Cha	Xe
22. *Astrolepis sinuata* (Lag. ex Sw.) D. M. Benham & Windham	CS,TS,MS	Ep	Cha	Xe
23. *Bommeria hispida* (Mett. ex Kuhn) Underw.	CS	Ep	Hem	Xe
24. *Cheilanthes allosuroides* (Mett.) Gruz & Windham	CS,TS	Ep	Cha	Xe
25. *Cheilanthes leucopoda* Link	MS, RS	Ep	Hem	Xe
26. *Cheiloplecton rigidum* var. *rigidum* (Sw.) Feé	TS	T	Hem	Xe
27. *Gaga hirsuta* (Link) Fay-Wei Li & Windham	QF,JS	T	Hem	Xe
28. *Gaga kaulfussii* (Kunze) Fay-Wei Li & Windham	CS	Ep	Hem	Xe
29. *Gaga purpussi* (T. Reeves) Fay-Wei Li & Windham	QF	T	Hem	Xe
30. *Mildella fallax* (M. Martens & Galeotti) Nesom	QF	T	Hem	Xe
31. *Myriopteris aemula* (Maxon) Gruz & Windham	RV	Ep	Cha	Xe
32. *Myriopteris aurea* (*Poir.*) *Gruz & Windham*	CS,TS,MS,RS	Ep	Cha	Xe
33. *Myriopteris cucullans* (Feé) Gruz & Windham	GF,QF,CS, JS,MS	Ep	Cha	Xe
34. *Myriopteris rufa* Fée	QF	Ep	Cha	Xe
35. *Myriopteris scabra* (Maxon) Gruz & Windham	TS	Ep	Cha	Xe
36. *Myriopteris jamaicensis* (Maxon) Gruz & Windham	MS	Ep	Hem	Xe
37. *Myriopteris lendigera* (Cav.) Fée	QF	T	Cha	Xe
38. *Myriopteris lindheimeri* (Hook.) J. Sm.	CS,TS	Ep	Hem	Xe
39. *Myriopteris marsupianthes* Fée	QS	Ep	Hem	Xe
40. *Myriopteris microphylla* (Sw.) Gruz & Windham	MS	Ep	Cha	Xe
41. *Myriopteris myriophylla* (Desv.) J. Sm.	QF,MS,RS	Ep	Cha	Xe

Appendix. Continued.

Taxonomic group	Vegetation type	Substrate	Life form	Foliar strategy
42. *Myriopteriss notholaenoides* (Desv.) Gruz & Windham J.Sm.	QF,MS,RS,RV	T	Hem	Xe
43. *Myriopteris pringlei* (Davenp.) Gruz & Windham sub. *moncloviensis* (Baker) Gruz & Windham	CS	Ep	Hem	Xe
44. *Myriopteris tomentosa* (Link) Fée	MS	Ep	Hem	Xe
45. *Myriopteris windhamii* Grusz	QF,CS,TS,QS	Ep	Hem	Xe
46. *Notholaena affinis* (Mett.) Hook. ex T. Moore	CS	Ep	Hem	Xe
47. *Notholaena aschenborniana* Klotzsch	CS,MS,RS	Ep	Cha	Xe
48. *Notholaena candida* (M. Martens & Galeotti) Hook	TS	Ep	Cha	Xe
49. *Notholaena galeottii* Feé	TS,MS	Ep	Cha	Xe
50. *Notholaena sulphurea* (Cav.) J. Sm.	TS,RV	Ep	Cha	Xe
51. *Pellaea atropurpurea* (L.) Link	MS	Ep	Cha	De
52. *Pellaea cordifolia* (Sessé & Moc.) A. R. Sm.	QF,CS	Ep	Hem	De
53. *Pellaea ovata* (Desv.) Weath.	TS,QS	Ep	Cha	De
54. *Pellaea ternifolia* subsp. *ternifolia* (Cav.) Link	CS,QS	Ep	Cha	Xe
55. *Pteris cretica* L.	GF	A	Hem	Me
56. *Pteris longifolia* L.	TS	T		
Aspleniaceae				
57. *Asplenium monanthes* L.	GF,QF	T	Hem	Me
58. *Asplenium resiliens* Kunze	QF,JS,MS,RV	T	Hem	Me
Thelypteridaceae				
59. *Thelypteris hispidula* (Decne.) C. F. Reed	GF	A	Cha	Me
60. *Thelypteris kunthii* (Desv.) C.V. Morton	MS	A	Cry	Me
61. *Thelypteris ovata* var. *lindheimeri* (C. Chr.)	CS,MS,RS,RV	T	Cry	Me
62. *Thelypteris pilosa* (M. Martens & Galeotti) Crawford	GF	Ep	Cha	Me
63. *Thelypteris puberula* var. *puberula* A. R. Sm.	RV	A	Cry	Me
Woodsiaceae				
64. *Cystopteris fragilis* (L.) Bernh.	GF,QF,JS	T	Hem	Me
65. *Woodsia mexicana* Fée	QF	Ep	Hem	Me
66. *Woodsia mollis* (Kaulf.) J. Sm.	QF	T	Cha	Me
Dryopteridaceae				
67. *Dryopteris cinnamomea* (Cav.) C. Chr.	QF	T	Hem	Me
68. *Dryopteris wallichiana* (Spreng.)	GF	T	Cha	Me
69. *Elaphoglossum potosianum* Christ	QF	T	Cha	De
Tectariaceae				
70. *Tectaria heracleifolia* (Willd.) Underw.	RV	T	Cha	Me
Polypodiaceae				
71. *Phlebodium pseudoaureum* (Cav.) Lellinger	CS,SV	Ep	Cha	De

Appendix. Continued.

Taxonomic group	Vegetation type	Substrate	Life form	Foliar strategy
72. *Pleopeltis guttatum* Maxon	QF	E	Cha	Po
73. *Pleopeltis madrensis* (J.Sm.) A.R. Sm. & Tejero	QF	E	Cha	Po
74. *Pleopeltis plebeia* (Schltdl. & Cham.) A.R. Sm. & Tejero	RV	E	Cha	Po
75 a. *Pleopeltis polylepis* (*Roemer ex Kunze*) *T. Moore* var. *interjecta* (Weath.) E. A. Hooper	GF	E	Cha	Po
75 b. *Pleopeltis polylepis* (*Roemer ex Kunze*) *T. Moore* var. *polylepis*	QF	E	Cha	Po
76. *Pleopeltis polypodioides* (L.) E.G. Andrews & Windham var. *polypodioides*	QF,TS, MS,QS,RV	E	Cha	Po
77. *Pleopeltis thyssanolepis* (A. Braun ex Klotzsch) E.G. Andrews & Windham	CS	Ep	Cha	Po
78. *Polypodium martensii* Mett.	QF	E	Cha	De
79. *Polypodium plesiosorum* Kunze	GF	T	Hem	De
80. *Polypodium subpetiolatum* Hook.	QF	T	Hem	De

Vegetation type: CS = Crassicaule scrub, GF = Gallery forest, JS = *Juniperus* scrub, MS = Microphyll scrub, QF = *Quercus* forest, QS = *Quercus* scrub, RS = Rosette scrub, RV = Riparian vegetation, SV = Secondary vegetation, TS = Thorny scrub. Substrate: E = Epiphytic, Ep = Epipetric, T = Terrestrial, A = Amphibious. Life form: Cha = Chamaephyte, Cry = Cryptophyte, Hem = Hemicryptophyte, Hy = Hydrophytes. Foliar strategy: Xe = Xeromorphic, De = Deciduous, Me = Mesomorphic, Po = Poikilohydric.

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Retypification of *Cheilanthes incisa* (Pteridaceae)

Pedro Bond Schwartsburd
Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Av. P.H. Rolfs s.n., Viçosa, MG, Brazil, CEP 36570-900, e-mail: pedro.schw@ufv.br
Jefferson Prado
Instituto de Botânica, C.P. 68041, CEP 04045-972, São Paulo, SP, Brazil, e-mail: jprado.01@uol.com.br

Abstract.—A neotype was chosen for *Cheilanthes incisa* by previous workers because there were apparently no extant syntypes. Recently, we found some extant isosyntypes in some European herbaria (L, LE, OXF, and PRC). Thus, we here select a lectotype for *C. incisa*, superseding the previous neotype. We also provide an updated synonymy.

Key Words.—*Asplenium fluminense*, Heinrich Karl Beyrich, August Gustav Heinrich Bongard, *Hypolepis incisa*, *Lonchitis tenuifolia*, nomenclature

Cheilanthes Sw. is currently recognized as a highly polyphyletic genus and many attempts of new circumscription of smaller genera are in progress (*e.g.*, Eiserhardt *et al.*, 2011; Grusz and Windham, 2013; Li *et al.*, 2012; Link-Perez *et al.*, 2011; Prado *et al.*, 2007). *Cheilanthes incisa* Kunze ex Mett. (provisionally kept in *Cheilanthes s.l.*) is a distinctive species but superficially similar to *Gaga* Li *et al.* and *Aspidotis* (Nutt. ex Hook.) Copel., due to the skeletonized lamina aspect and reduced laminar tissue. It is especially similar to *Aspidotis* and to *C. shimperi* Kunze (also provisionally kept in *Cheilanthes s.l.*), due to the mucronate ultimate segments, but shows substantial differences in microscopic features, such as epidermal cells and spores (Ponce *et al.*, 2007). *Cheilanthes incisa* is a narrow endemic to the mountains of Rio de Janeiro, southeastern Brazil, and shows no apparent relationship to any other Brazilian *Cheilanthes* (Mynssen and Windisch, 2002; Ponce *et al.*, 2007). The species was validly published by Mettenius (1859) and later neotypified by Ponce *et al.* (2007). It has one heterotypic synonym: *Hypolepis serrata* Fée.

Ponce *et al.* (2007) chose to neotypify *Cheilanthes incisa* because they did not find any extant syntypes. At the time, it was thought that all syntypes had been deposited at LZ and destroyed during WW II (see Stafleu and Cowan, 1979, 1981, regarding information about Kunze and Mettenius). Based on the protologue, Ponce *et al.* (2007) believed that the type collection came from Serra da Estrella, Rio de Janeiro (Brazil). Thus, they chose as the neotype *A. C. Brade 16288* (RB, image seen; isoneotype: SI), which was collected in that region.

The main objective of the present paper is to revise this previous neotypification based on new data we gathered recently from European herbaria.

65. *Ch. incisa* Kz. herb.

Rhizoma tenerum repens caespitosum vel oblique adscendens, paleis subulatis fuscis rigidulis vestitum; folia chartacea laete viridia glabra; petiolus 1—2″ longus, fuscus nitidus; lamina 1—1¾″ longa, ovata bi-tripinnatisecta; segmenta primaria utrinque 3—6, erecto-patentia petiolata, 8‴ longa, ovato-oblonga, ultima laxe disposita, e basi cuneata spathulato-lanceolata, submucronato-acuminata, inciso-serrata; dentes infimi plerumque steriles acuti superiores plerumque margine externo monosori abbreviati retusi; nervi indivisi, steriles basin dentium intrantes, superiores fertiles in dorso abbreviato retuso dentium soriferi; lobuli reflexi herbacei vel pallidi, transverse oblongi vel elongati, integerrimi (Fig. 28—31).

Asplenium fluminense Bong. herb. Kz.; — Lonchitis tenuifolia Beyr.

Rio-Janeiro; Sierra D'Estrella.

Differt a Ch. californica, cui proxima, laciniis ultimis plerumque margine extrorso monosoris, lobulis indusiiformibus transverse lunato-oblongis vel elongatis, sorisque ad apices retusos dentium abbreviatorum sitis; nec minus dispositione segmentorum secundi et tertii ordinis infimis, nempe plerumque latus exterius laminae occupantibus, ab Ch. Schimperi et californica diversa.

Fig. 1. Protologue of *Cheilanthes incisa* from Mettenius (1859).

Methods

We visited the following herbaria searching for syntypes of *Cheilanthes incisa* and its synonyms: FI, G, K, L, LE, OXF, PR, and PRC. Images of materials from the following herbaria were observed online: B, K, P, and RB.

Results and Discussion

An analyses of Mettenius' protologue (1859:44; Fig.1), plus a new search in European herbaria, lead us to conclude there are, in fact, extant isosyntypes of *Cheilanthes incisa*. Thus, according to McNeill *et al.* (2012:Art. 9.19(a)), we here supersede the neotype chosen by Ponce *et al.* (2007), and select a lectotype.

In the protologue (Fig. 1), Mettenius (1859) cited the species as "*Ch. incisa* Kz. herb.", clearly validating Kunze's unpublished name. Below the description, he cited "*Asplenium fluminense* Bong. herb. Kz.", and "*Lonchitis tenuifolia* Beyr.", and then "Rio-Janeiro; Sierra D'Estrella". Thus the specimens he examined personally were from Rio de Janeiro and Serra da Estrella. On the other hand, he implicitly cited two collections in the protologue. The first was a specimen annotated by A. G. H. Bongard as "*Asplenium fluminense*", present in Kunze's herbarium (LZ), and now certainly destroyed. The second was a specimen annotated by H. K. Beyrich as "*Lonchitis tenuifolia*". These two collections are the syntypes of *Cheilanthes incisa*.

According to Stafleu and Cowan (1976) and Stafleu and Mennega (1993), Beyrich's herbarium was later incorporated in BM, LZ (now destroyed), and Z. Duplicates of his collections may be found in other herbaria such as B, GOET, H, LE, M, MO, NY, OXF, PH, and WRSL. During our search in European herbaria, we found some specimens of "*Lonchitis tenuifolia* Beyrich," one of

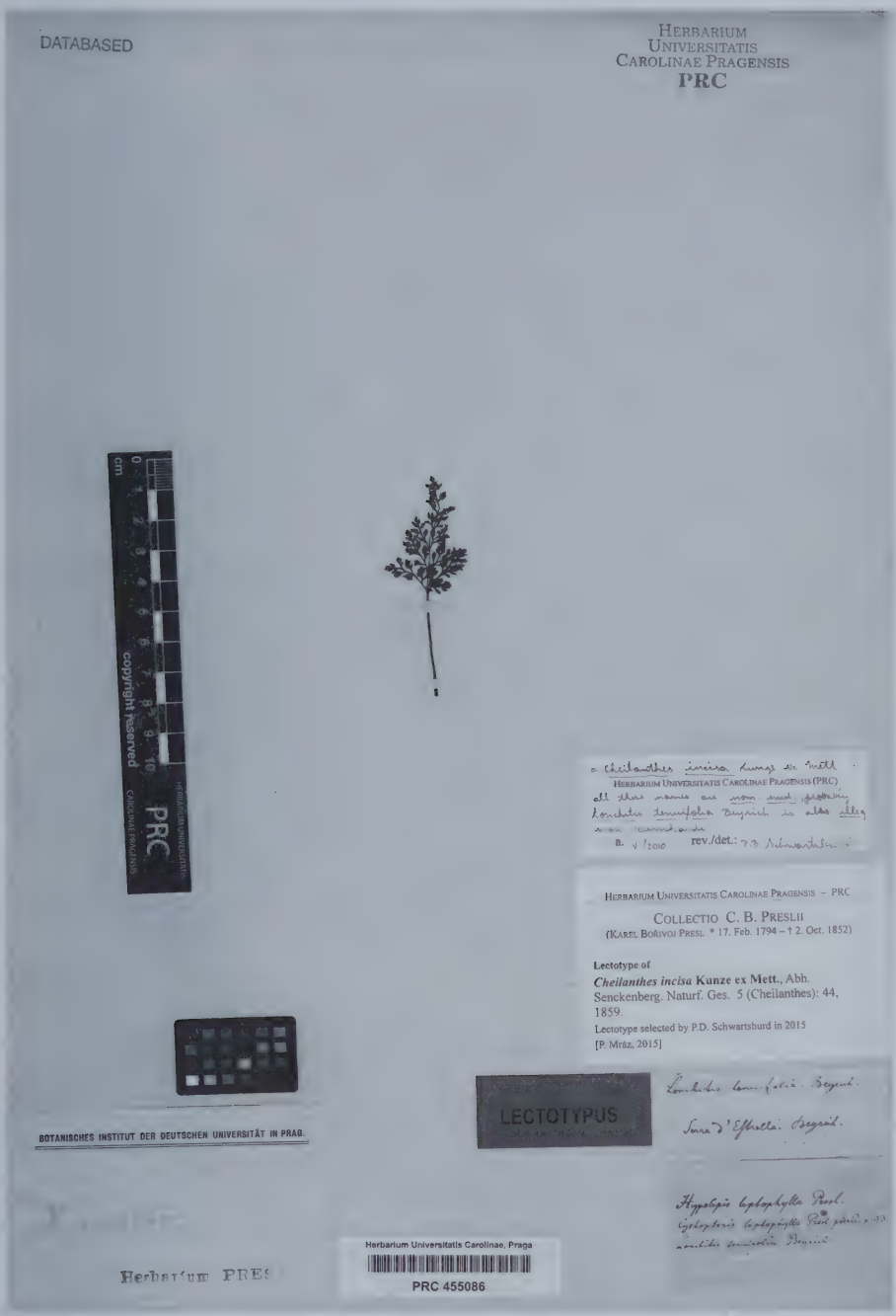

FIG. 2. Scanned image of the lectotype of *Cheilanthes incisa* (*Beyrich s.n.* [PRC]). Reproduced by kind permission of PRC.

the syntypes of *Cheilanthes incisa*. Thus, we here choose one specimen (Fig. 2) as lectotype for *C. incisa* and its duplicates as isolectotypes, as follow:

Cheilanthes incisa Kunze ex Mett., Farngatt. 5 (*Cheilanthes*): 44, n. 65, tab. 3, Figs. 28–31. 1859. *Hypolepis incisa* (Kunze ex Mett.) C. Chr., Index Fil. 371. 1905. LECTOTYPE (designated here).—BRAZIL. [Rio de Janeiro]: Sierra d'Estrella, 1822–1823, *H. K. Beyrich s.n.* (PRC!; isolectotypes: L! [annotated as "*Lonchitis asplenioides*"], LE! [ex Herb. Chamisso]). **Figs. 1, 2**

Hypolepis serrata Fée, Cr. Vasc. Br. 1: 53, tab. 13, Fig. 3. 1869. LECTOTYPE (designated by Ponce *et al.* 2007:144).—BRAZIL. **Rio de Janeiro:** *A. Glaziou 2336* (K, image seen; isolectotypes: B-200037160, image seen, HBG, image seen, P-00271010, image seen, P-00271012, image seen).

Cystopteris leptophylla C. Presl, Tent. Pterid. 93. 1836, *nom. nov.* for *Lonchitis tenuifolia* Beyrich, *nom. nud. et illeg.* (non *Lonchitis tenuifolia* G. Forst., 1786). *Hypolepis leptophylla* (C. Presl) C. Presl, Epimel. Bot. 66. 1851, *nom. nud.*

The material below may also represent remaining isosyntypes of *Cheilanthes incisa,* but not seen by the author. Probably, duplicates of any of these were sent by Bongard (in LE) to Kunze (in LZ), and later analyzed by Mettenius (in LZ). Due to the lack of information on the labels and in the protologue, it is impossible to establish which exact material(s) Mettenius had real contact (*Riedel 92* seems more likely, due to the annotation on the labels).

BRAZIL. [Rio de Janeiro]: Mandioca, s.d., *L. Riedel 92* (LE! [annotated as "*Asplenium fluminense*", "*Cheilanthes incisa*"]); Mandioca, 1822, *L. Riedel 61* (LE!); Mandioca, 1822, [*L. Riedel*] *s.n.* (LE! [ex Herb. Acad. Sci. Petropol.]); locality unknown, s.d., *G. H. Langsdorff & L. Riedel 190* (LE!); locality unknown, s.d., *L. Riedel s.n.* (LE! [ex Herb. Petropol.]); locality unknown, s.d., *L. Riedel 89* (LE!); locality unknown, s.d., *L. Riedel s.n.* (OXF! [ex Herb. Prescot]).

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Erratum

AFJ volume 105 issue 2, pp. 101–112 (April – June 2015)

Figure 6 in the *American Fern Journal* article entitled ***Isoëtes* Kamchatka (northern Russian Far East), with the Description of a New Hybrid *I.*×*paratunica* (*I. asiatica*×*I. maritima*)** by Olga A. Mochalova, Alexander A. Bobrov, and Daniel F. Brunton was originally published incorrectly. The figure was incorrectly duplicated on page 109 and should have only appeared once on page 108.

INFORMATION FOR AUTHORS

Authors are encouraged to submit manuscripts pertinent to the study of ferns and lycophytes for publication in the *American Fern Journal*. Manuscripts should be sent to the editor at **hauk@denison.edu.** Acceptance of papers for publication depends on merit as judged by two or more referees. Authors are encouraged to contribute toward publishing costs; however, the payment or non-payment of page charges will affect neither the acceptability of manuscripts nor the date of publication. Mandatory fees will be charged for color figures in print and/or in electronic versions of published papers.

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